Regulation of ColE1 plasmid DNA replication in *Escherichia coli*

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To my mother

for making it all possible

and

to my wife and daughter

for making it all worthwhile

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Abstract

A number of plasmid copy-number mutants have been characterized in order to gain insight into the mechanism by which initiation of plasmid DNA synthesis is regulated. One mutant, pFH118, which has a 12-fold higher copy number than the homologous wildtype plasmid, RSF1050, contains 16 additional base pairs within a region of the plasmid that has been shown to be essential for normal replication. The insertion mutation lies approximately 500 base pairs upstream of the origin of DNA synthesis within the coding region of a small untranslated RNA, RNA I. The copy-number mutation is recessive and can be complemented both in *cis* and in *trans* by the wild-type RNA I gene. The results of the complementation studies reveal that replication of the ColE1-type plasmids is regulated by a mechanism of negative control in which RNA I functions as a repressor of plasmid DNA synthesis.

The target of RNA I inhibition is believed to be near the 5' end of a second plasmid transcript, RNA II. RNA II transcription begins 555 base pairs upstream of the replication origin and terminates several hundred base pairs downstream of the origin. RNA II can be processed by RNase H to generate a primer for the initiation of DNA synthesis by DNA polymerase I. Purified RNA I has been shown to inhibit primer formation in vitro. Dominant high copy-number mutants of a specially-constructed plasmid, pDM247, contain single base-pair changes within a seven base-pair sequence of the RNA II coding region. The altered nucleotides in RNA II are

located within the single-stranded loop of a prominent hairpin structure that can form near the 5' end of the transcript. A complementary hairpin structure exists in RNA I. A model for RNA I-RNA II interaction involving base pairing of loop structures has been proposed.

A single base-pair mutation has been identified in a plasmid which has a temperature-sensitive high copy-number phenotype. The ts mutant exhibits normal copy control in cells growing at the permissive temperature of 30°C but replicates uncontrollably in cells shifted to the nonpermissive temperature, 37°C. Runaway replication of plasmid DNA is lethal to the host cells within 3-4 hours after the temperature shift. The orp mutation (for over-replication) affects both the RNA II transcript and the promoter for RNA I. RNA I promoter-galK fusion studies indicate that the mutation does not create a temperature-sensitive promoter. It has been proposed that the orp mutation creates a thermosensitive secondary structure in RNA II. The breakdown of this structure at the higher temperature renders the plasmid insensitive to replication inhibition by RNA I. Secondary mutations which suppress the temperature-sensitive phenotype have been isolated. These mutations also affect nucleotides within RNA II.

A small plasmid-encoded polypeptide also plays a role in plasmid copy number control. Although this protein, referred to as the rop gene product, is not essential for regulation of plasmid DNA replication (the deletion of the rop gene causes only a three to

five-fold increase in plasmid copy number), its presence in the cell can suppress lethal DNA over-replication of the temperature-sensitive copy number mutant. This suppression was used as the basis of a selection for rop insensitive mutants. The identification of point mutations within the RNA I coding region of these mutants is consistent with the hypothesis that the product of the rop gene modulates the interaction between RNA I and the primer precursor.

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Introduction

The stable inheritance of genetic information in bacteria requires a mechanism that ensures the complete replication of all genetic elements, or replicons, during each cell generation. Although much progress has been made in the enzymology of DNA replication, the molecules that regulate initiation of DNA replication are largely unknown. Extrachromosomal elements, because of their small size and genetic dispensibility, provide attractive model systems for investigating the molecular mechanisms of replication control. These elements include the bacteriophages (e.g., lambda, P1 and ØX174) and plasmids (e.g., F factor, ColE1, and pSC101). Since plasmids are normally maintained at defined copy numbers in the cell, they provide a more suitable model for the study of replication control than the bacteriophages which during their lytic stage of development replicate their DNA in a seemingly uncontrolled manner. The bacterial plasmids have been categorized as stringent or relaxed on the basis of their copy number. The stringent plasmids are maintained at one to two plasmid molecules per host chromosome. Plasmids belonging to this class include the F factor and the drug resistance plasmids, R6-5, R1 and R100. The relaxed plasmids are present in greater amounts, typically between 10-30 copies per chromosome. Plasmids of this class share a number of common replication features including a dependence on DNA polymerase I and the ability to continue replication in the absence of protein synthesis. This latter property allows the accumulation of very large amounts of plasmid DNA (>1000 copies per cell) when a

protein synthesis inhibitor, such as chloramphenicol or spectinomycin, is added to a culture of plasmid-containing cells (Clewell, 1972). Consequently, these plasmids are often used to provide cloning vectors, because of the increased yield of material.

Replication of the relaxed plasmids, despite the implication of the name, is controlled in a strict manner, giving a stable average number of plasmid molecules per cell in an exponentially growing population of bacteria cells. This implies the existence of a copy control mechanism that ensures one complete doubling of plasmid molecules during each cell generation. Several models have been proposed to account for the stable maintenance of plasmid replicons during cell growth and division. The replicon model of Jacob, Brenner and Cuzin (1963), a positive control model, postulates that replication and maintenance of replicons takes place on cell membrane attachment sites. Replication occurs when the sites are duplicated and progeny molecules are subsequently distributed among daughter bacteria at cell division. A model for negative control of replication proposed by Pritchard, Barth and Collins (1969), postulates that a replication repressor is involved in the control of initiation of plasmid DNA synthesis. Replication is initiated when the plasmid-specified repressor is diluted out to a subcritical concentration during the course of cell growth. Plasmid replication increases the number of plasmid molecules in the cell, and also increases the number of active repressor genes. New initiation of plasmid DNA synthesis is inhibited when the critical concentration

of the repressor is obtained. Consequently, this model is generally referred to as the Inhibitor Dilution Model. A number of experiments indicate that replication of most plasmids is mediated by a negatively-acting control element and is therefore consistent with this model.

The colicin E1 plasmid of Escherichia coli (ColE1) is considered the prototype of the relaxed multicopy plasmids, normally existing at 10-15 copies per chromosome. ColE1 is a closed circular DNA molecule (approximately 6600 base pairs) that determines the production of an inducible antibiotic protein, colicin E1, and confers immunity to colicin E1 upon its host (Bazaral and Helinski, 1970). ColE1 DNA can be replicated in vitro using a soluble enzyme system. No plasmid-encoded proteins are required for ColE1 replication either in vitro or in vivo (Donoghue and Sharp, 1978; Kahn and Helinski, 1978). ColE1 DNA synthesis is initiated from a unique origin site on the plasmid with replication proceeding unidirectionally around the plasmid genome (Tomizawa et al., 1977). All of the information necessary for maintenance of ColE1 as a plasmid lies within a 580 base pair fragment derived from the origin region (Backman et al., 1978; Oka et al., 1979). The origin of DNA synthesis is located only 13 base pairs from one end of this fragment, suggesting that little or no information is necessary downstream from the origin. The 580 bp origin fragment contains two start sites for transcription by RNA polymerase in vitro (Itoh and Tomizawa, 1980). Transcription from one of these sites located

about 450 nucleotides away from the origin proceeds in the direction opposite to replication, producing a small transcript, approximately 100 bases in length (Levine, 1978; Levine and Rupp, 1978; Morita and Oka, 1979). This transcript has been designated as species I RNA or simply RNA I. Transcription from the other site produces a transcript, RNA II, that can be processed by RNase H to form a primer for initiation of DNA synthesis in vitro (Itoh and Tomizawa, 1980).

The picture of ColE1 DNA replication control that has recently emerged from the results of mutational studies described here and those of others (Conrad and Campbell, 1979; Muesing et al., 1981; Lacatena and Cesareni, 1981), along with the in vitro experiments by Tomizawa and coworkers (Tomizawa et al., 1981; Tomizawa and Itoh, 1981; Tomizawa and Itoh, 1982), is that ColE1 DNA replication is regulated at the level of initiation by the concentration of RNA I in the cell. RNA I is unstable and constitutively transcribed so that its cellular concentration is directly related to the number of plasmid molecules in the cell. Initiation of plasmid replication is inhibited when the RNA I concentration exceeds some critical level. Cell growth and division dilutes the concentration of RNA I and plasmid molecules allowing new initiation of plasmid DNA synthesis. The inhibitory role of RNA I appears to be mediated through a base-pairing interaction with RNA II which prevents the utilization of the latter transcript as a replication primer (Cesareni, 1981; Lacatena and Cesareni, 1981).

ColE1 copy number is also influenced by a small plasmid-encoded protein, the rop gene product (Cesareni et al., 1982; Som and Tomizawa, 1983). Although this protein is not essential for the replication or stable maintenance of ColE1, the deletion of the rop gene increases the copy number of the plasmid approximately five-fold (Twigg and Sherrat, 1980). While the function of the rop protein remains largely unknown, there is increasing evidence that this element reduces plasmid copy number by modulating the interaction between RNA I and RNA II (see Chapter 3).

The mechanism by which ColE1 regulates its copy number in the cell, is also responsible for the plasmid's incompatibility properties (Tomizawa and Itoh, 1981; Moser and Campbell, 1983). Incompatibility, defined as the inability of two similar plasmids to be stably maintained in the same cell, results when the negative regulator of plasmid replication (RNA I) is unable to distinguish between two homologous replicons (Novick and Hoppensteadt, 1978).

In Chapter 1 of this thesis I describe the characterization and complementation of a mutant high copy-number derivative of the multicopy plasmid, pMB1. The results of this study confirm the role of RNA I as the negatively-acting regulatory element that is responsible for both plasmid copy-number control and incompatibility. In Chapter 2 I identify a single base pair substitution within the RNA II coding region of a temperature-sensitive plasmid copy mutant. This mutation causes lethal plasmid over-replication at the nonpermissive temperature presumably by

creating a thermosensitive secondary structure in RNA II near the site of RNA I inhibition. Chapter 3 outlines a selection for plasmid mutants with alterations in the region where the rop gene product mediates its effect upon plasmid copy number. Finally, in Chapter 4, I present genetic studies which implicate the central loop in RNA II as the site where RNA I initially interacts in its inhibition of primer formation.

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Chapter 1

Characterization and Complementation of a pMB1 Copy-Number Mutant: Effect of RNA I Gene Dosage on Plasmid Copy Number and Incompatibility

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Characterization and Complementation of pMB1 Copy Number Mutant: Effect of RNA I Gene Dosage on Plasmid Copy Number and Incompatability

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A 16-base-pair insertion has been identified as the mutation responsible for the high-copy-number phenotype of the plasmid copy number mutant pFH118. The mutation is located near the plasmid origin of replication in a region of the genome that encodes two overlapping RNA transcripts. One of these transcripts, RNA I, acts as a negative regulator of plasmid replication. The second transcript is the precursor to the primer for the initiation of DNA synthesis. We demonstrate through complementation that the pFH118 DNA overproduction phenotype is a consequence of the reduced effectiveness of the mutant RNA I at inhibiting plasmid replication and not a consequence of an altered target site on the primer precursor. In addition, a series of plasmids containing multiple RNA I-coding genes was constructed for investigating the effects of RNA I gene dosage on plasmid copy number and incompatibility. The results of this study strongly support the inhibitor dilution model of plasmid copy control with RNA I as the plasmid-specified inhibitor responsible for both copy number control and incompatibility.

The stable inheritance of multicopy bacterial plasmids at defined copy numbers implies a mechanism that strictly regulates the number of plasmid replication events within each cell division cycle. The inhibitor dilution model (19, 20) proposes that initiation of plasmid DNA replication is repressed by a plasmid-specified, *trans*-acting element, whose cellular concentration reflects the concentration of plasmids in the cell. Plasmid replication increases the number of plasmid molecules and the number of active inhibitor genes inside the cell. When a "critical" level of inhibitor is obtained, initiation of plasmid DNA synthesis is completely repressed. During growth and division of the cell, the inhibitor is diluted below the critical concentration, permitting new initiation of plasmid DNA synthesis.

For the amplifiable plasmids containing the replicons of ColE1, pMB1, NTP1, p15A, or C1oDF13, there is accumulating evidence that a small, replicon-specified transcript, RNA I, functions as the primary inhibitor of plasmid DNA replication (6, 18, 25, 26). We previously proposed the involvement of RNA I in plasmid copy number control from our observation that the insertion mutation responsible for the mutant high-copy phenotype of the pMB1-derived plasmid, pFH118, lies within the coding region for this small transcript (6). Spontaneous revertants of pFH118, displaying the wild-type copy number, were found to have deleted the insertion sequence and produced a normal RNA I. Tomizawa et al. (26) later demonstrated that purified RNA I inhibits initiation of plasmid DNA synthesis in vitro by preventing the processing of a second plasmid transcript to form an RNA primer for DNA polymerase I. The primer precursor is initiated about 550 nucleotides upstream from the origin of DNA synthesis and terminates downstream of the origin (13). Processing of this transcript involves RNase H, an endonuclease which preferentially degrades RNA in RNA-DNA hybrids. RNA I, through an undefined mechanism, interacts with the primer precursor during its transcription to prevent the transcript from forming a stable RNA-DNA hybrid with its template near the replication origin (14, 25).

RNA I and the primer precursor are both transcribed from the same region of the plasmid genome, but from opposite DNA strands (13). One consequence of the transcripts' overlap is that the entire RNA I nucleotide sequence is complementary to the 5′ end of the primer precursor. The RNA I molecule has a high degree of internal sequence symmetry (17) and may, under normal physiological conditions, adopt a tRNA-like structure with three partially double-stranded regions and three single-stranded loops (designated by Roman numerals I, II, and III in Fig. 1b). The active sites of the RNA I

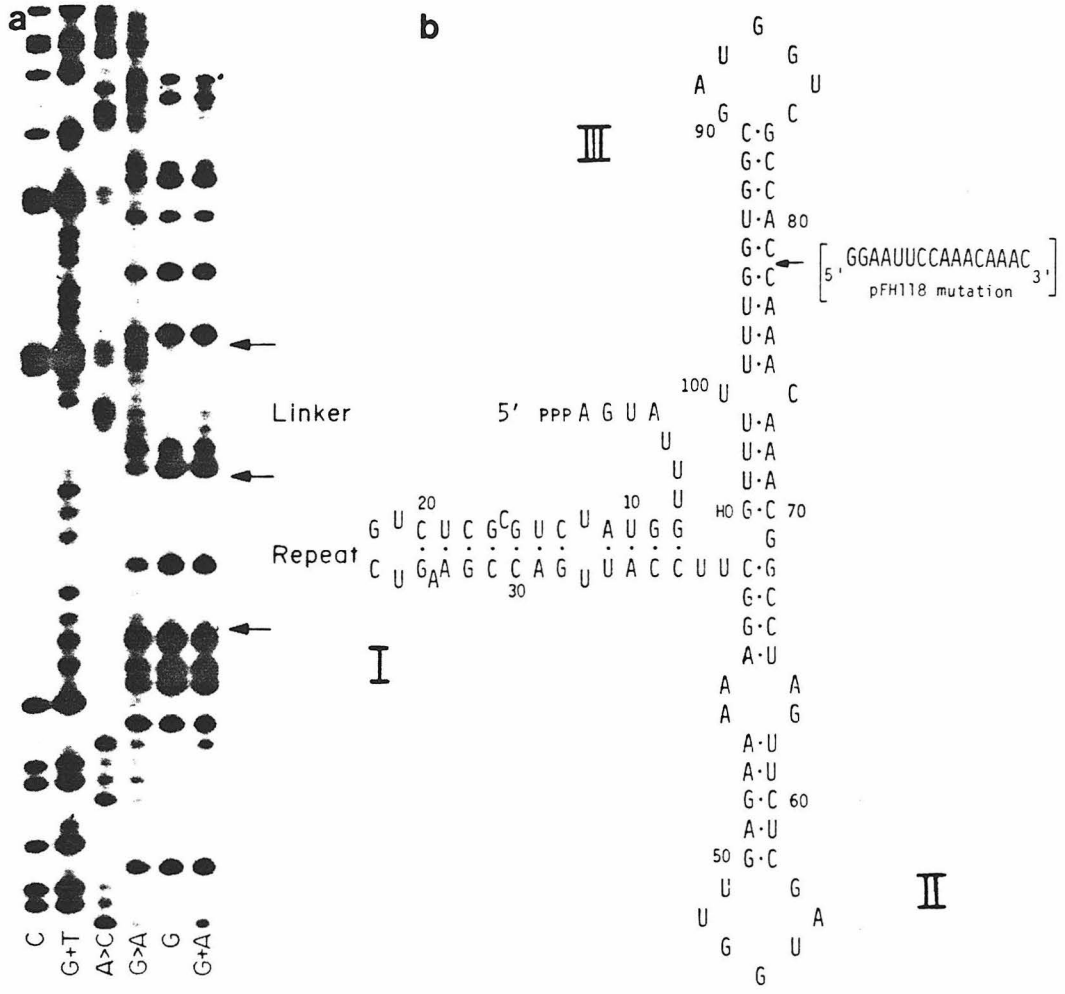

FIG. 1. (a) Representative sequencing gel of the RNA I-coding region of the copy number mutant pFH118. Sequencing was done by the method of Maxam and Gilbert (16). Details are provided in the text. The nucleotides bracketed by arrows are the additional nucleotides introduced into RSF1050 during mutagenesis. (b) Nucleotide sequence of RNA I from pFH118 as deduced from our DNA sequences. The secondary structure of the wild-type RNA shown is that proposed by Morita and Oka (17). The location of the insertion in pFH118 is indicated by the arrow, and the sequence of the additional nucleotides is presented in brackets. With the exception of this insertion, the nucleotide sequence of this region was found to be identical to that of pBR322 and pMB9, also derivatives of pMB1, and to differ from that of ColE1 at one position (24, 25).

molecule, like the active site of the tRNA, are considered to lie within one or more of the single-stranded loops (9, 14, 25). A similar stem-and-loop structure can also be predicted for the region of the primer precursor complementary to RNA I. From characterizations of inhibitor target mutants of pMB1, Lacatena and Cesareni (14) conclude that the secondary structure of this region of the primer precursor is required for successful inhibitory interaction. Sequence analysis of these mutants reveals the importance of the central loop (loop II) in the mechanism for plasmid copy number control.

In this paper we present results of complementation experiments with a high-copy-number mutant of pMB1 from which we conclude that loop structure III of RNA I is required for the transcript's inhibitory function. A 16-nucleotide insertion within the stem of this structure severely reduces the effectiveness of the transcript as a repressor of plasmid replication. The homologous insertion within the primer precursor does not appear to inactivate the target of inhibition. We also present evidence of a "critical" concentration of RNA I (as deduced from gene dosage) for complete repression of plasmid repli-

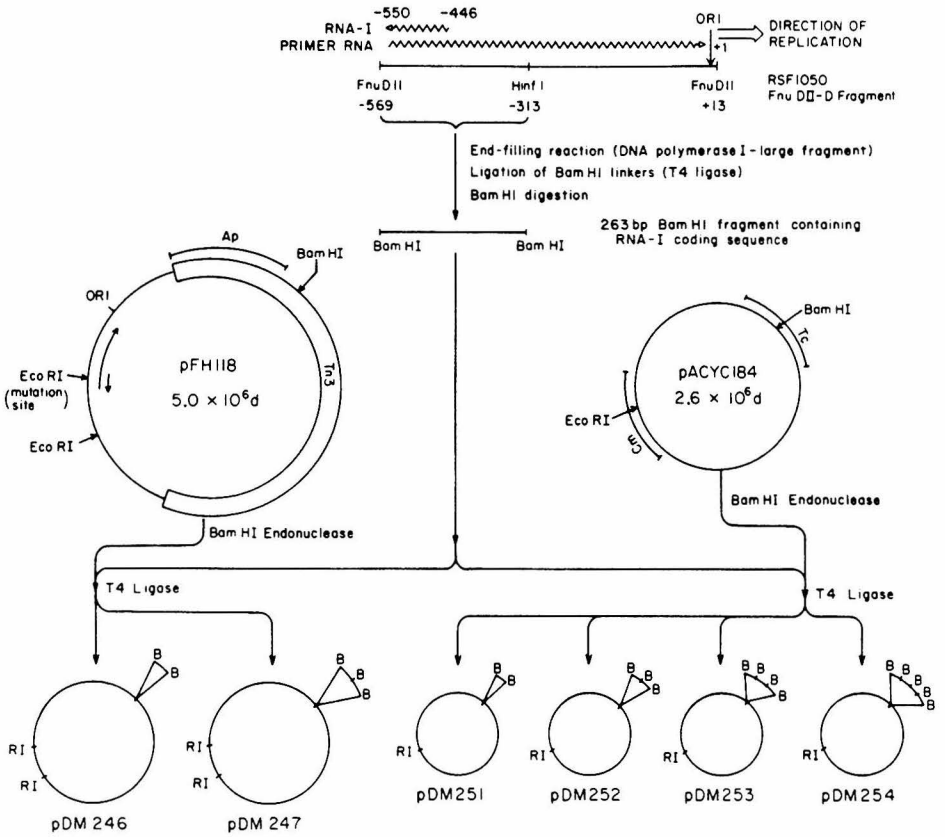

FIG. 2. Construction of plasmids. The 582-bp *Fnu*DII-D fragment of RSF1050 was cleaved with *Hin*fI to yield two fragments. The *Hin*fI ends of the fragments were made blunt ended by repair with DNA polymerase I. *Bam*HI ends were generated by attachment of *Bam*HI linkers followed by digestion with *Bam*HI endonuclease. The DNA fragments were separated from the unattached linkers by Bio-Gel A-5m (Bio-Rad Laboratories) chromatography and then ligated with *Bam*HI-digested pFH118. The ligation mixture was used to transform *E. coli* HB101. Ampicillin-resistant transformants were screened by restriction enzyme analysis of plasmid DNA from minilysates. Recombinant plasmids containing one and two 263-bp *Bam*HI inserts were isolated and designated pDM246 and pDM247, respectively. Purified inserts from *Bam*HI-digested pDM247 DNA were partially polymerized by allowing the inserts to self-ligate in the presence of T4 ligase. *Bam*HI-cut pACYC184 was then added, and ligation was continued. After transformation and screening of chloramphenicol-resistant, tetracycline-sensitive colonies, clones containing pACYC184 derivatives with one, two, three, and four *Bam*HI inserts were isolated. These plasmids were designated pDM251 through pDM254, respectively.

cation as postulated in the inhibitor dilution model. Finally, we present results which directly link plasmid copy-number control and incompatibility.

MATERIALS AND METHODS

Materials. *Bam*HI linkers (octamers) were obtained from Collaborative Research, Inc. Restriction enzymes were purchased from New England Biolabs. *Escherichia coli* DNA polymerase I (large fragment) was obtained from Boehringer Mannheim. Phage T4 polynucleotide ligase and kinase and *E. coli* RNA polymerase holoenzyme were gifts from Charles C. Richardson. [α-^{32}P]UTP (25 Ci/mmol) was from ICN Pharmaceuticals Inc.

Bacterial strains and plasmids. Experiments were carried out in *E. coli* HB101 *pro gal hsdR hsdM recA1*. Plasmid RSF1050 and its high-copy-number derivative, pFH118, have been described previously (12). Plasmid pACYC184 is a tetracycline- and chloramphenicol-resistant derivative of the cryptic miniplasmid p15A (3). Other plasmids used in these studies are described in Fig. 2 and Table 1.

Preparation of plasmid DNA. A modification of the method of Clewell and Helinski (4) was used to prepare plasmid DNAs and has been described previously in detail (5).

Agarose gel electrophoresis. Conditions for agarose gel electrophoresis have been previously described (7).

DNA sequencing. The 580-base-pair (bp) *Fnu*DII restriction fragment containing the origin region of RSF1050 (Fig. 2) was isolated from an acrylamide gel

TABLE 1. Plasmids used in this study

Plasmid	Replication origin	No. of copies[a]		Relative copy no.
		pMB1 RNA I-wt	pMB1 RNA I-mut	
RSF1050	RS1050 (wild type)	1		1
pFH118	pFH118 (mutant)		1	12
pDM246	pFH118	1	1	0.4
pDM247	pFH118	2	1	0.2
pDM248	pFH118		2	2.3
pACYC184	pACYC184			0.3
pDM251	pACYC184	1		0.3
pDM252	pACYC184	2		0.3
pDM253	pACYC184	3		0.3
pDM254	pACYC184	4		0.3

[a] The copy number of each plasmid was normalized to the amount of RSF1050, which was given a value of 1.

and labeled at the 5′ ends with T4 polynucleotide kinase and [γ^{32}P]ATP (21). This fragment was then cleaved with *Hae*III, generating a 430-bp fragment and a 150-bp fragment containing the RNA I-coding sequences. The nucleotide sequence of the 150-bp fragment was determined by the method of Maxam and Gilbert (16).

In vitro RNA synthesis. Covalently closed, circular plasmid DNA was transcribed by using purified *E. coli* RNA polymerase holoenzyme in a reaction mixture (0.1 ml) containing 50 mM Tris-hydrochloride (pH 7.9), 0.1 M KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg of yeast tRNA per ml, 40 μM each of four ribonucleotide triphosphates (including [α-^{32}P]UTP), 5 U of RNA polymerase per ml, and 10 μg of plasmid DNA per ml. Incubation was at 37°C for 30 min. The reaction was terminated by addition of 5 μl of 250 mM EDTA. After precipitation with ethanol, transcription products were separated by electrophoresis in a denaturing polyacrylamide gel containing 7 M urea (15).

RESULTS

Nucleotide sequence of the copy number mutation in pFH118. The plasmid RSF1050 is a 5.0 × 10^6-dalton, ampicillin-resistant derivative of the plasmid pMB8 into which Tn*3* has been transposed (10). pMB8 is a miniderivative of the naturally occuring plasmid pBM1, which is closely related to, but not identical with, ColE1 (1, 22). The high-copy-number mutant plasmid pFH118 was generated from RSF1050 by a technique that employs synthetic oligonucleotide *Eco*RI linkers as mutagens (12). The mutant plasmid contains a new *Eco*RI site at the site of linker insertion. In pFH118 this site is located about 520 bp upstream (5′ to the direction of replication) from the origin of replication in the coding region for the RNA I transcript (6).

To determine the exact location of the linker insertion in pFH118, the nucleotide sequence of the RNA I-coding region was determined. A representative sequencing pattern and sequence of RNA I as deduced from the DNA sequence we have determined is shown in Fig. 1. The *Eco*RI octamer used to construct the mutant is inserted 57 bp from the RNA termination site. In addition, eight base pairs 5′ to this insertion are repeated on the 3′ side, making the total size of the insertion 16 bp. This repeat was probably generated from the in vitro mutagenesis procedure, in which linear plasmids with single-stranded ends are repaired with DNA polymerase I before the addition of *Eco*RI linkers. Similar structures have been reported among Tn*3* mutants prepared by this procedure (11). In vitro transcription of pFH118 in the presence of RNase H reveals that both RNA I and the primer RNA are 16 nucleotides longer than the corresponding transcripts from RSF1050 (data not shown). The 16 additional nucleotides fall within stem III of the proposed secondary structures of RNA I and its complementary region in the primer precursor.

Characterization of the pFH118 mutation: complementation by the wild-type RNA I. Since the mutation in pFH118 falls within the region of the plasmid that encodes the inhibitor RNA I and also the target site of RNA I inhibition on the primer precursor, the plasmid's high-copy-number phenotype could be attributed to either a defective inhibitor or a modified target site that is no longer recognized by the inhibitor or both. In the first case, the addition of wild-type RNA I should result in the complementation of the high-copy-number mutation. We decided to investigate this possibility by inserting the wild-type RNA I coding sequence into a nonessential region of the copy number mutant pFH118. To avoid ambiguities related to copy number effects mediated by plasmid regulatory elements other than RNA I, such as that identified by Twigg and Sherratt (27), we used a small restriction fragment which contains little more than the essential nucleotide information for transcription of the complete wild-type RNA I. A 256-bp *Fnu*DII-*Hin*fI restriction fragment from RSF1050 contains the complete wild-type RNA I-coding sequence plus 21 bp of DNA 3′ to the

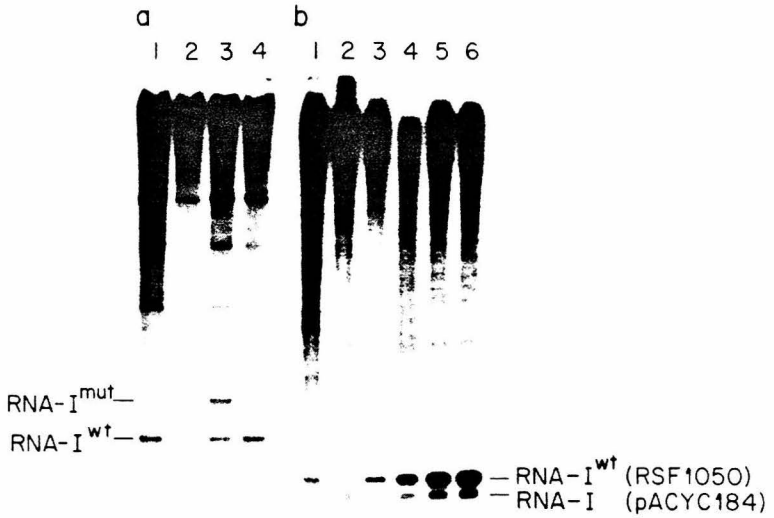

FIG. 3. In vitro synthesis of RNA I. Supercoiled plasmid DNA was transcribed in vitro as described in the text. ^{32}P-labeled transcripts were separated on either a 6% (a) or 8% (b) polyacrylamide gel (19:1 acrylamide/bisacrylamide ratio) containing 7 M urea. Gels were dried and autoradiographed as described previously (7). Plasmids transcribed were as follows: in a, (1) RSF1050, (2) pFH118, (3) pDM246, (4) pDM247; in b, (1) RSF1050, (2) pACYC184, (3) pDM251, (4) pDM252, (5) pDM253, (6) pDM254.

RNA I termination site and 130 bp of DNA 5′ to the transcript's initiation site, which includes the entire recognition sequence for RNA polymerase (Fig. 2 and Table 1). The fragment was inserted, by using *Bam*HI linkers, into the single *Bam*HI restriction site of pFH118, approximately 2.7 kb from the site of the copy number mutation. The recombinant plasmid, pDM246, when transcribed in vitro, produces two distinct RNA I transcript bands of approximately equal intensity which comigrate on a denaturing acrylamide gel with the RNA I transcripts of pFH118 (RNA I-mut) and RSF1050 (RNA I-wt) (Fig. 3a). A second recombinant which had picked up two *Bam*HI inserts was also isolated from cells transformed with the same ligation mixture. In vitro transcription of this plasmid, pDM247, yields both RNA I-wt and RNA I-mut transcripts at a ratio of approximately 2:1 (Fig. 3a). To test whether the wild-type RNA I transcribed from these plasmids complemented the high-copy-number mutation, we determined plasmid copy numbers.

Plasmid copy numbers were first examined in a general way from the relative resistances of plasmid-containing cells to ampicillin and then in a more quantitative way by direct analysis of plasmid DNA from cell lysates on agarose gels. Uhlin and Nordstrum (28) have shown for R1 plasmid derivatives that single cell resistance to ampicillin in agar plates is proportional to β-lactamase gene dosage. In Fig. 4, we compare ampicillin resistances of *E. coli* HB101 containing various plasmids. Bacterial cells which carry the high-copy-number mutant pFH118 are relatively insensitive to high levels of ampicillin up to 1,000 μg/ml, whereas bacteria carrying the homologous wild-type plasmid, RSF1050, are unable to grow at this drug concentration. Cells containing pDM246 are much more sensitive to lower ampicillin concentrations than is pFH118. This result suggests that the presence of the RNA I-wt gene complements the mutation in this plasmid, that is, that RNA I-wt binds better than RNA I mut to the pFH118 target. Cells containing plasmid pDM247 were the most sensitive to ampicillin, indicating that when two RNA I-wt genes are present, plasmid copy number is reduced even more. As a control, the RNA I-mut gene was isolated from pFH118 and inserted into the pFH118 *Bam*HI site. This plasmid, pDM248, is identical to pDM246, except that it carries the 16-bp insertion mutation within the cloned RNA I gene. Cells carrying pDM248, like cells carrying the high-copy number mutant pFH118, grow well on plates containing 1,000 μg of ampicillin per ml.

To confirm that plasmid copy number effects

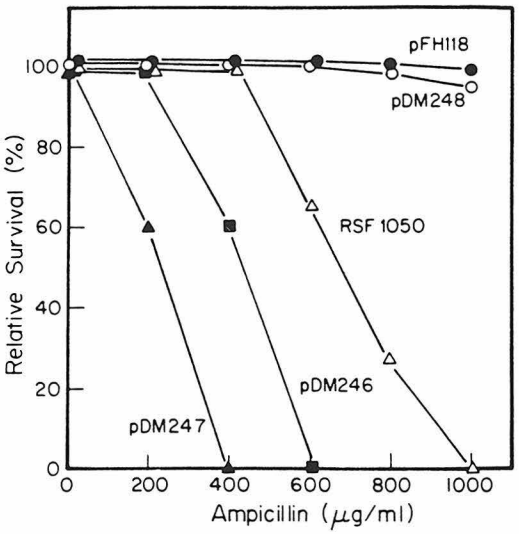

FIG. 4. Ampicillin resistance of various plasmid-containing cells. *E. coli* HB101 organisms containing indicated plasmids were taken from 5-ml overnight cultures in L broth supplemented with 0.2% glucose and spread onto L plates containing 0.2% glucose and various amounts of ampicillin. Visible colonies were counted after an 18-h incubation at 37°C. Relative survival is determined as a percentage of the colonies growing on plates without ampicillin.

were responsible for the observed differences in cellular resistance to ampicillin, quantitative determinations of plasmid copy number were made from electrophoretic analysis of plasmid DNA from cell lysates as described in the legend to Fig. 5. The plasmid copy numbers obtained with this procedure correlate well with the relative ampicillin resistances of cells containing these plasmids. These results are summarized in Table 1 and indicate that (i) the copy number of pFH118, as a result of the 16-bp insertion mutation within the RNA I coding region, is 12-fold higher than that of the homologous wild-type plasmid RSF1050; (ii) insertion of a 256-bp restriction fragment encoding the wild-type RNA I into pFH118 (pDM246) results in a 30-fold reduction of the plasmid copy number; (iii) insertion of a second RNA I-wt gene into this plasmid gives a further twofold copy number reduction (pDM247). In contrast, insertion of the mutant RNA I gene into the pFH118 *Bam*HI site gives only a fivefold reduction of copy number (pDM248). The copy number of plasmid pDM248 is still more than two times greater than that of the wild-type plasmid, RSF1050. From these observations we conclude that the plasmid overproduction phenotype of pFH118 results from the inability of RNA I-mut to effectively inhibit plasmid DNA replication. The mutant RNA I, however, is not completely ineffective as an inhibitor of replication (compare copy numbers of plasmids pFH118 and pDM248 containing one and two RNA I genes, respectively). The reduced effectiveness of this transcript in repressing initiation of plasmid DNA synthesis probably results from the disruption of the secondary structure of the RNA molecule by the 16

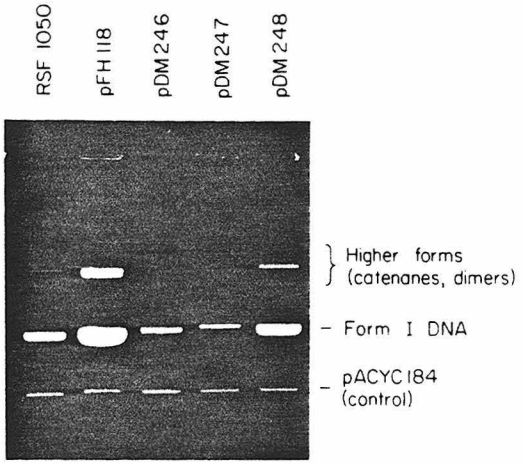

FIG. 5. Plasmid copy number comparison. *E. coli* HB101 cells containing plasmids RSF1050, pFH118, pDM246, pDM247, or pDM248 were grown in 150 ml of L broth containing 0.2% glucose and 25 μg of ampicillin per ml to an optical density at 550 nm between 0.6 and 0.9 and quickly chilled on ice. After dilution of the cultures to an optical density 0.6, 150 ml of each culture was mixed with an equal volume of a similarly diluted culture of HB101 cells containing the plasmid pACYC184. The addition of the pACYC184-containing cells was to provide an internal standard for monitoring the recovery of plasmid DNA after cell lysis. Cleared cell lysates were then prepared by the procedure of Clewell and Helinski (4), and supercoiled plasmid DNAs were isolated by equilibrium centrifugation in cesium chloride-ethidium bromide density gradients. The plasmid DNAs were then analyzed by electrophoresis through a 0.9% agarose gel. The lower band in each lane contains the supercoiled form of pACYC184. The upper bands contain the supercoiled forms of the pMB1-derived plasmids. The relative amounts of plasmid DNA were quantitated by densitometry. A photographic negative of another gel (not shown) containing different dilutions of these samples was scanned to ensure that comparative measurements of band densities were done within the linear range of the densitometer. Areas under the densitometry peaks were integrated with the aid of a Tektronix 4965 digitizing tablet interfaced to a Tektronix 4052 computer. By using the previously reported value of 18 for the minimal copy number of pACYC184 in HB101, the minimal copy numbers of the pMB1 plasmids were calculated to be as follows: RSF1050, 60; pFH118, 720; pDM246, 24; pDM247, 12; pDM248, 140.

additional nucleotides in stem III. Complementation of the pFH118 mutation by the wild-type RNA I indicates that the target of inhibition is not severely altered by the 16-nucleotide insertion within the primer precursor. This implies that stem structure III of the primer precursor is not of primary importance for this mechanism of copy number control.

RNA I and incompatibility. Two closely related plasmids are said to be incompatible if they are unable to stably coexist within the same bacterial cells growing under nonselective conditions. As cells which originally carry two incompatible plasmids divide, either one of the plasmids will be lost from the cell population or else the cells may segregate into two populations, each containing only one of the two plasmids. According to the inhibitor dilution model, incompatibility arises as a consequence of the inability of the plasmid-specified inhibitors to distinguish their respective replicons (20). Tomizawa and Itoh (25) have isolated several ColE1 mutants with altered incompatibility properties. These incompatibility mutants contain single-base-pair changes within the RNA I coding sequence. In vitro transcription experiments demonstrate that the single-base-pair changes affect both the ability of RNA I to inhibit primer formation and the sensitivity of primer formation to inhibition by RNA I. Each of the incompatibility mutants they examined was also a high-copy-number mutant, implicating RNA I as the plasmid-specified element involved in both copy number control and incompatibility.

We decided to further investigate incompatibility effects due to RNA I inhibition by inserting the pMB1 RNA I gene into a plasmid compatible with pMB1. The RNA I coding region from the pMB1-derived plasmid, RSF1050, was ligated into the single *Bam*HI restriction site of pACYC184 (Fig. 2). Plasmid pACYC184 contains the replicon from p15A and is compatible with ColE1-pMB1-derived plasmids (3). To compare the effects of RNA I gene dosage on incompatibility, four recombinant plasmids were constructed which contain from one to four identical pMB1-RNA I genes inserted into the pACYC184 *Bam*HI site. The chimeric plasmids, pDM251 through pDM254, each code for the transcription of two distinct species of RNA I (Fig. 3b). The slightly longer RNA I is identical to that transcribed from RSF1050. The smaller RNA I is transcribed from pACYC184.

We first examined the effects of the chimeric plasmids on the high-copy-number mutant pFH118. We attempted to prepare doubly transformed cells by introducing pFH118 by transformation into *E. coli* HB101 carrying pACYC184 or one of the chimeric plasmids, pDM251 through pDM254, and selecting on L agar plates containing chloramphenicol (25 μg/ml) and ampicillin (50 or 1,000 μg/ml). The results of this transformation are presented in Table 2. Cells containing pACYC184 were efficiently transformed to resistance to 50 μg of ampicillin per ml with pFH118. These double transformants also grew well on plates containing 1,000 μg of ampicillin per ml, indicating that pFH118 was present at high copy number. In contrast, transformation frequencies (from plates with 50 μg of ampicillin per ml) of cells containing any of the chimeric plasmids were appreciably lower and decreased with each additional pMB1 RNA I gene carried by the resident plasmid. With three or four of these genes present, no transformants with pFH118 could be obtained. If the resident plasmid contained only one or two of these genes, transformants with pFH118 could be isolated from plates with 50 μg of ampicillin per ml, but not from plates with 1,000 μg of ampicillin per ml, indicating that the copy number of pFH118 is reduced in these cells.

These results indicate that the pFH118 high-copy-number mutation is complemented by the pMB1 species of RNA I. In addition, we observe a correlation between the number of RNA I genes carried by the resident plasmid and the transformation frequency with pFH118 as the incoming plasmid. The most striking aspect of these results is the failure of pFH118 to become established in cells where there are more than two pMB1 RNA I genes on the resident plasmid. We interpret these results as indicating that the elevated level of RNA I in these cells at the time

TABLE 2. Transformation of various plasmid-carrying strains with pFH118 (Ap)[a]

Resident plasmid	No. of pMB1 RNA I coding regions	Transformants per μg of pFH118 DNA appearing on plates containing ampicillin	
		50 μg/ml	1,000 μg/ml
pACYC184 (Cm, Tc)	0	1.1×10^6	8.7×10^5
pDM251 (Cm)	1	6.0×10^5	$<10^3$
pDM252 (Cm)	2	2.0×10^5	$<10^3$
pDM253 (Cm)	3	$<10^3$	$<10^3$
pDM254 (Cm)	4	$<10^3$	$<10^3$

[a] HB101 cells carrying the indicated plasmids were transformed with 20 ng of pFH118 after a standard transformation procedure (8). Each transformation mixture was added to 2 ml of L broth and incubated at 37°C for 2 h before plating only L agar plates containing 0.2% glucose and the antibiotics chloramphenical (25 μg/ml) and ampicillin (50 μg/ml). After incubation for 18 h at 37°C the visible colonies were counted. A transformation frequency of $<10^3$/μg means that no transformants were observed.

TABLE 3. Exclusion of pFH118 and RSF1050 from cells after the establishment of a second plasmid encoding pMB1 RNA I[a]

Incoming plasmid	No. of pMB1 RNA I genes per plasmid	Fraction of total cells after 6 generations exhibiting ampicillin resistance	
		pFH118	RSF1050
pACYC184	0	1.0	1.0
pDM251	1	1.0	1.0
pDM252	2	0.8	1.0
pDM253	3	$<10^{-3}$	$<10^{-3}$
pDM254	4	$<10^{-3}$	$<10^{-3}$

[a] The plasmids indicated were used to transform *E. coli* HB101 carrying either pFH118 or RSF1050. Colonies which grew up on agar-plates containing both ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml) were used to prepare cultures of cells growing in L broth supplemented with 0.2% glucose. After approximately six generations of growth at 37°C under nonselective conditions, samples of the cultures were spread onto L plates and L plates containing ampicillin (50 μg/ml). After overnight incubation of the plates at 37°C the colonies were counted, and the ratio of ampicillin-resistant cells to total cells was determined.

of pFH118 introduction completely inhibits replication of the incoming plasmid, thus preventing its establishment. In agreement with this interpretation we found that doubly transformed cells of pFH118 and each of the chimeras could be prepared by beginning with cells containing pFH118 and then introducing one of the chimeras by transformation. All of the doubly transformed cells prepared in this manner could be selected initially on plates containing chloramphenicol and 1,000 μg of ampicillin per ml.

Colonies from these plates were used to conduct a segregation test (Table 3). After six generations of growth in L broth in the absence of selective drugs, bacteria were spread onto L plates without drugs and L plates containing 50 μg of ampicillin per ml. We found that pFH118 was not excluded from the cells which also carried pACYC184 or the chimeras containing one or two pMB1 RNA I genes. However, pFH118 was rapidly lost from cells carrying the chimeras with three or four pMB1 RNA I genes. When segregaton tests were conducted with RSF1050 instead of pFH118, similar results were obtained, with RSF1050 being rapidly excluded only by pDM253 and pDM254.

These results can be explained according to the inhibitor dilution model. Since the p15A replicon of pACYC184 is not inhibited by the pMB1 RNA I species (26), the copy numbers of plasmids pDM251 through pDM254 are approximately equal to the copy number of pACYC184 (data not shown). We have observed that pACYC184 is maintained at a copy number that is approximately one-third that of the pMB1-derived plasmid, RSF1050 (Fig. 5). If the cellular concentration of RNA I is proportional to the number of RNA I genes, as postulated by the inhibitor dilution model, then the pMB1 RNA I concentration in cells containing the chimeric plasmids will be lower than the critical inhibitory concentration unless there are at least three copies of the gene transcribing this RNA per pACYC184 vector. The abrupt change in compatibility properties that we observe in going from two to three pMB1 RNA I genes per vector probably results from the increase in cellular RNA I concentration to a level exceeding the critical inhibitory concentration.

DISCUSSION

Perhaps the most powerful technique for investigating the molecular mechanisms responsible for the regulation of DNA synthesis in *E. coli* is the generation and characterization of plasmid copy number mutants. In this paper we extend our previous studies on the high-copy-number mutant pFH118 by identifying the mutation as a 16-bp insertion within the region of the plasmid genome which codes for the transcription of RNA I and the primer precursor. The mutation disrupts nucleotide pairing within the stem of one of three loop structures in the proposed secondary structure for both RNA I and the 5′ end of the primer precursor. We demonstrate through complementation studies that the mutation in pFH118 affects the ability of RNA I to inhibit plasmid replication without noticeably affecting the sensitivity of the plasmid to inhibition by wild-type RNA I. The pFH118 high-copy-number phenotype is suppressed when a 256-bp DNA fragment that codes for the transcription of the wild-type RNA I is cloned directly into pFH118 or into a second, unrelated plasmid within the same cell. The location of the mutation within the stem of the loop structure III (Fig. 1) suggests that this structure is critical for the inhibitory function of RNA I, but is not essential within the primer precursor for target site recognition. Lacatena and Cesareni (14) characterized dominant target mutations that were not complemented by wild-type RNA I. Their results demonstrated the importance of the central loop (loop II) in both RNA I and the primer precursor for successful inhibition. They have also found a dominant target mutation within the stem of loop I. Muesing et al. (18) have reported a copy number mutant of ColE1 that contains a single base-pair alteration within the stem of loop III. This mutation, like the mutation in pFH118, can be complemented in *trans* by the wild-type plasmid (23).

According to the inhibitor dilution model, plasmid copy number is regulated through a

mechanism of feedback inhibition. Plasmid replication increases the number of plasmids per cell, which in turn increases the concentration of the plasmid-specified inhibitor. Plasmid replication ceases when the level of the inhibitor reaches a "critical" level for the complete repression of initiation of plasmid DNA synthesis. One would predict from this model that if the number of inhibitor molecules produced from each plasmid molecule were doubled, the stable copy number of the plasmid would be reduced to half of its original value. In agreement with this prediction and with the evidence implicating RNA I as the plasmid-specified inhibitor of replication, we have observed that plasmid pDM247, which contains two identical wild-type RNA I genes, has a copy number which is one-half that of plasmid pDM246, which contains only a single wild-type RNA I gene.

We have also presented evidence directly linking RNA I inhibition to the phenomena of plasmid incompatibility, a finding previously reported by Tomizawa and Itoh (25). The plasmid pACYC184, which is normally compatible with the pMB1-derived plasmid, RSF1050, and its copy number mutant, pFH118, exhibits severe incompatibility toward these plasmids when more than two pMB1 RNA I genes are inserted into the plasmid's single *Bam*HI site. The rapid exclusion of RSF1050 and pFH118 from cells containing either pDM253 or pDM254 implies that the former pMB1-derived plasmids are unable to replicate in the presence of the latter plasmids containing three and four pMB1 RNA I genes. Transcription of RNA I from these plasmid genes is probably sufficient to maintain an RNA I level above the "critical" concentration for full inhibition of plasmid DNA synthesis from the pMB1 replicon.

Although it now seems clear from our work and the work of others that inhibition of plasmid DNA replication by RNA I is an important part of the mechanism for regulating plasmid copy number, there is evidence that other plasmid-encoded elements may be involved in copy control. Twigg and Sherratt (27) have shown that deletion of a nonessential region of ColE1 gives a derivative with an elevated copy number. The deletion mutation is complementable by a product encoded by the *Hae*II-C restriction fragment of ColE1. Cesareni et al. (2) have recently identified a gene within this fragment which encodes a 63-amino-acid polypeptide which they have designated rop (for repressor of primer). They proposed that the product of this gene regulates plasmid replication by repressing the transcription of the primer for DNA synthesis. Unlike RNA I, this product does not appear to be involved in determining plasmid incompatibility.

ACKNOWLEDGMENTS

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Chapter 2

Suppressors of a Temperature-Sensitive Copy-Number Mutation in Plasmid NTP1

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Suppressors of a Temperature-Sensitive Copy-Number Mutation in Plasmid NTP1

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Summary. A temperature-sensitive high copy-number mutant of plasmid NTP1, first described by Grindley et al. (1978), is lethal to bacterial cells at the non-permissive temperature. This behavior has been used to select mutations in the plasmid replication origin region that suppress the over-replication phenotype. We have identified the site of the original ts lethal mutation and the positions of the reversion mutations. The ts mutation, designated *orp*, for over-replication, is a single nucleotide change 23 base-pairs upstream from the transcription start site for RNA I, the repressor of plasmid replication. This change simultaneously affects the promoter for RNA I and the precursor of the primer for plasmid replication, RNA II, which is also transcribed from this region. Fusions of the mutant promoter region with the *galK* gene indicate that transcription is not temperature sensitive. This result suggests that the mutation affects RNA II secondary structure. The reversion mutations are also located within the RNA II coding region more than 200 bp from the site of the original ts mutation. These mutations may also affect RNA II structure.

Introduction

The plasmid NTP1 is a small, multicopy plasmid which exhibits a number of replication features in common with ColE1. These features include a strict requirement for RNA polymerase and DNA polymerase I and the ability to undergo amplification in the presence of protein synthesis inhibitors such as chloramphenicol (Yamada et al. 1979; Crosa et al. 1975; Conrad et al. 1979). Recent evidence suggests that NTP1 is identical to the plasmid RSF1030 (Yamada et al. 1979; Conrad et al. 1979; Grindley and Nakada 1981; Som and Tomizawa 1982). NTP1 and ColE1 exhibit extensive sequence homology over a region of approximately 550 bp that begins near the origin of replication (defined as the site of initiation of unidirectional DNA synthesis) and extends upstream from the origin (away from the direction of replication) (Grindley and Nakada 1981; Som and Tomizawa 1982; Tomizawa et al. 1981). This region encodes two transcripts that are involved in regulating the frequency of initiation of DNA replication from the plasmid origin. One of these transcripts, RNA I, is about 100 nucleotides long. It initiates from a promoter approximately 100 bp upstream of the plasmid origin and is transcribed in the direction opposite to the direction of DNA synthesis (Som and Tomizawa 1982; Tomizawa et al. 1977; Morita and Oka 1979). The second transcript, RNA II, is transcribed from the DNA strand complemetary to that encoding RNA I (Itoh and Tomizawa 1980). RNA II initiates a few nucleotides beyond the termination site of RNA I and terminates downstream of the origin of replication. The entire sequence of RNA I is complementary to the 5′-end of RNA II.

RNA I acts as a replicon-specific repressor of replication, and is considered to be an important part of the mechanism of plasmid copy-number control and plasmid incompatibility (Conrad and Campbell 1979; Moser and Campbell 1983; Muesing et al. 1981; Tomizawa et al. 1981; Tomizawa and Itoh 1981; Lacatena and Cesareni 1981). In the absence of RNA I, RNA II may form an RNA-DNA hybrid with its template near the origin (Itoh and Tomizawa 1980; Tomizawa et al. 1981). This structure serves as a substrate for RNase H which cleaves the RNA in the hybrid at the origin (Itoh and Tomizawa 1980). The new 3′ end of the transcript functions as a primer for the initiation of DNA synthesis by DNA polymerase I. Interaction of RNA I with its complementary region in RNA II inhibits primer formation, probably by preventing the formation of the RNA-DNA hybrid (Tomizawa et al. 1981).

There is accumulating evidence that the secondary structure of the nascent RNA II chains is important for primer formation. Tomizawa and Itoh (1982) have recently reported results of *in vitro* transcription pausing experiments which indicate that the interaction of RNA I with its complementary region on the nascent RNA II transcript alters the folding of this transcript further downstream affecting the formation of the hybrid structure at the origin. In this paper we present additional evidence supporting the role of RNA II secondary structure in the regulation of initiation of plasmid replication. We have been studying a temperature-sensitive high copy-number mutant of NTP1 that was originally described by Grindley et al. (1978). At the restrictive temperature, runaway replication of the mutant plasmid, pJN75, is lethal to the host cell. We have taken advantage of this conditional lethality to select for revertants of the mutant plasmid. We have identified the original ts copy-number mutation in pJN75 as well as a number of secondary-site mutations which suppress the ts mutant copy-number phenotype. The ts mutation is a single base pair change within a region of the plasmid that encodes RNA II and also functions as the promoter for transcrip-

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tion of RNA I. This mutation is similar in nature to the mutations previously described by Wong et al. (1982) which lie within the RNA I promoter of several temperature-sensitive copy-number mutants of ColE1. Promoter fusion studies on the RNA I promoters of the ts mutant derivatives of both ColE1 (Wong et al. 1982) and NTP1 (this work) indicate that the mutations do not create temperature-sensitve promoters. Rather, the ts phenotype of these mutants may be a consequence of a thermosensitive secondary structure within RNA II. The secondary mutations which suppress the ts mutant phenotype of pJN75 also occur within the RNA II coding region more than 200 bp away from the site of the original ts mutation.

Materials and Methods

Bacterial Strains and Plasmids. Escherichia coli K12 derivatives HB101 and N100 have been described previously (Conrad and Campbell 1979; McKenny et al. 1981).

NTP1 is an ampicillin-resistant, nonconjugative plasmid which was isolated from *Salmonella typhimurium* (Yamada et al. 1979). NTP1 appears to be identical to the independently isolated plasmid RSF1030 (Yamada et al. 1979; Conrad et al. 1979; Grindley and Nakada 1981; Som and Tomizawa 1982).

Plasmid pNG77 is a temperature-sensitive copy-number mutant derived from NTP1 by nitrosoguanidine mutagenesis (Grindley et al. 1978). Plasmids pJN70 and pJN75 are deletion derivatives of NTP1 and pNG77, respectively, and contain only the *Hae*II A and E fragments of the parental plasmids (Grindley and Nakada 1981).

Plasmid pKO1 is described in McKenny et al. (1981) and Table 1.

Construction of Plasmids Containing RNA I Promoter-galK Fusions. A 233 bp *Alu*I fragment of pJN70 contains the promoter and transcription initiation site for RNA I. This fragment, or the homologous fragment from pJN75, was ligated to *Sma*I cleaved pKO1 and the ligation mixture was used to transform *E. coli* N100 to ampicillin resistance. Transformants were screened for the presence of recombinant plasmids by gel electrophoretic analysis of plasmid DNA from cell lysates. Derivatives of pK01 containing single inserts of the promoter fragment in each orientation were isolated.

Mutagenesis of pJN75 and Selection of Revertants of pJN75. A culture of *E. coli* HB101 containing pJN75 was grown at 30° C to an A_{590} of 0.3 in 5 ml of L broth. Chloramphenicol (100 μg/ml) and N-methyl-N'-nitro-N-nitrosoguanidine (20 μg/ml) were added, and incubation was continued overnight. Plasmid DNA was isolated from the cells and introduced into *E. coli* HB101 by the transformation procedure of Dagert and Ehrlich (1979). Six temperature-resistant transformants were isolated from the mutagenized cells, while none were obtained without mutagen treatment. Plasmid DNA was prepared from isolated colonies and analyzed by agarose gel electrophoresis. Each revertant plasmid appeared to be identical in size to pJN75. When the purified plasmid DNAs were again introduced into strain HB101 cells by transformation, the transformants obtained were also viable at 37° C.

DNA Sequencing. DNA sequences of pJN75 and the temperature-resistant revertant plasmids were determined according to the method of Maxam and Gilbert (1980). The region that was sequenced in each plasmid extends from position −560 upstream of the origin to position +173.

Table 1. Expression of galactokinase *in vivo* from RNA I promoter-*galK* gene fusions

Plasmid	RNA I promoter	Promoter orientation	Galactokinase units	
			30° C	39° C
pKO1	none		4	7
pKO1-70	pJN70	+	3,472	4,904
pKO1-75	pJN75	+	1,664	2,472
pKO1-07	pJN70	−	11	11
pKO1-57	pJN75	−	11	10

Promoter fragments were inserted into the *Sma* I site of the pKO1 vector located upstream of the *galK* gene. Promoter orientations were determined from restriction enzyme analysis. A plus sign indicates that the promoter is oriented toward the *galK* gene while a minus sign (−) indicates that the promoter is oriented away from the *galK* gene. Plasmid-containing N100 cells were grown at 30° C or 39° C in M-56 medium supplemented with 0.3% fructose and 0.1% casamino acids to log phase ($O.D._{650}$ = 0.3 to 0.6). Galactokinase activitiy was determined by the procedure of McKenny et al. (1981). Galactokinase units are expressed as nmol of galactose phosphorylated per min per ml of cells at $O.D._{650}$ of 1.0. The copy numbers of pKO1-70 and pKO1-75 are approximately four-fold lower than the copy number of pKO1 (data not shown). The galactokinase units shown for pKO1-70 and pKO1-75 have been normalized to the copy number of pKO1

Results

Identification of the Mutation Responsible for the ts Plasmid Over-Replication Phenotype. The plasmid pNG77 is a temperature-sensitive copy-number mutant of NTP1 (Grindley et al. 1978). Bacteria containing the plasmid pNG77 grow normally at the permissive temperature (30° C), but cease growth and die one to two generations after a shift to the nonpermissive temperature (37° C). Loss of viability appears to be a consequence of "runaway" replication which results in an increase in plasmid synthesis from about 8% of chromosomal DNA synthesis to 150% in the first 10 min after the temperature shift (Grindley et al. 1978). Plasmids pJN75 and pJN70 are smaller (5.4 kb) deletion derivatives of pNG77 and NTP1, respectively (Grindley and Nakada 1981).

The nucleotide sequence surrounding the NTP1 origin of replication is known (Grindley and Nakada 1981; Som and Tomizawa 1982). We have compared the nucleotide sequences of pJN70 and pJN75 over a region extending from a *Hinc*II site 560 bp upstream from the origin of plasmid DNA synthesis of the *Hae*II site located 176 bp downstream of the origin. We have identified a single base pair difference at position −398 relative to the plasmid origin (Fig. 1). The mutation of pJN75 is a GC to AT transition which we have designated *orp* for plasmid overreplication. Subcloning experiments described below support our inference that this nucleotide change causes the ts mutant replication behavior of pJN75. The *orp* mutation changes a G in RNA II to an A approximately 130 nucleotides from the 5′ end of the transcript. In addition, this mutation alters the nucleotide sequence within the promoter for RNA I.

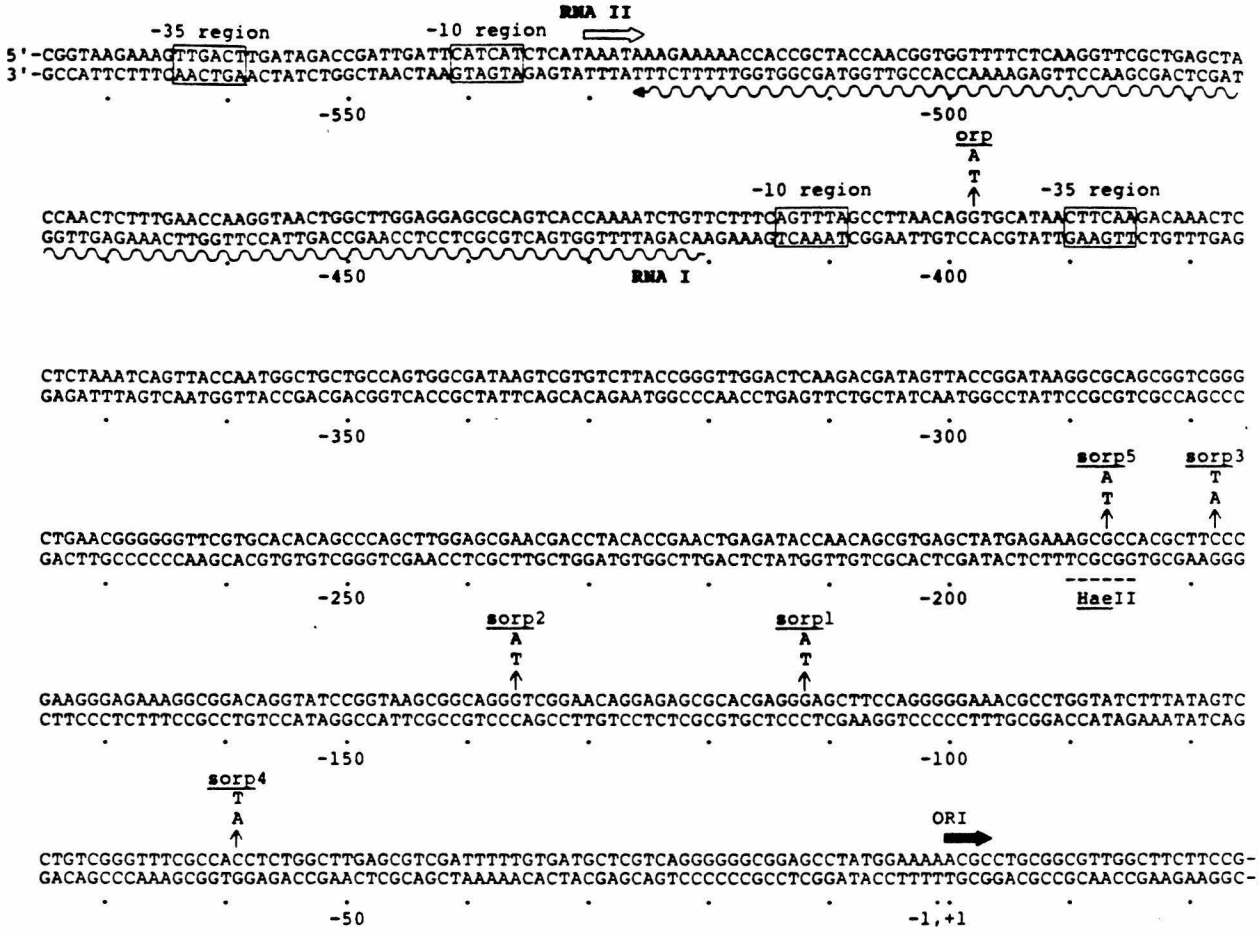

Fig. 1. DNA sequence of the replication origin region of NTP1. The nucleotide sequence shown is that of NTP1 near its replication origin (Grindley and Nakada 1981; Conrad et al. 1979). This sequence has recently been shown to be identical to the origin sequence of RSF1030 (Som and Tomizawa 1982). Nucleotide positions are numbered relative to the origin of DNA synthesis (ori). Position +1 is defined as the site of incorporation of the first deoxyribonucleotide during plasmid DNA replication (Som and Tomizawa 1982). The orientation of replication is indicated by the dark solid arrows. The RNA I coding region is shown by the long wavy arrow (Som and Tomizawa 1982; Oka et al. 1979). The white arrow indicates the start site and direction for RNA II transcription. The −10 and −35 promoter recognition sequences for the two transcripts are identified by boxes (Som and Tomizawa 1982). The *orp* mutation at position −398 gives rise to a temperature-sensitive plasmid over-replication phenotype that is lethal to the host cell (Grindley et al. 1978). This phenotype is suppressed by any one of the *sorp* mutations. The identification of the *orp* and *sorp* mutations is described in the text

The mutation occurs 23 bp upstream of the RNA I transcription start site (Som and Tomizawa 1982).

Analysis of RNA I Promoter Strength Using Promoter-galK Gene Fusions. To determine if the *orp* mutation creates a temperature-sensitive RNA I promoter, we analyzed relative promoter strengths as deduced from fusions of the mutant and wild-type promoters with the structural gene for galactokinase. For these studies we constructed derivatives of the plasmid vector pKO1 which contains the *galK* structural gene from *E. coli*. Galactokinase expression from this vector is dependent upon the insertion of an exogenous promoter upstream of the translational start site (McKenny et al. 1981, and Table 1). A 233 bp *AluI* restriction fragment containing the RNA I promoter from either pJN70 or pJN75 was inserted into the *SmaI* site of pK01. Recombinant plasmids containing single promoter fragments inserted in both possible orientations were used to transform N100 cells to ampicillin resistance. Plasmid-containing cells were then grown at either 30° C or 39° C and extracts were assayed for galactokinase activity as described in the legend to Table 1. As shown in Table 1, the mutation in the pJN75-derived fusion reduces galactokinase activity to about 50% of that observed with the wild-type pJN70 insert, regardless of the temperature at which the cells are grown. However, there is no significant difference in the response to temperature of the two promoters by these criteria. Both mutant and wild-type promoter inserts give about 1.5 times as much galactokinase activity at 39° C as at 30° C. We infer from these data that RNA I promoter function is not temperature sensitive in pJN75 and that the conditional high copy number is not likely to be attributable to a temperature-dependent reduction in the amount of RNA I transcription in the cell.

Isolation of Temperature-Resistant Revertants of pJN75. Since the *orp* mutation does not appear to affect the RNA I promoter in a temperature-dependent manner, the ts mutation may affect the RNA II directly. The conditional lethality of pJN75 enables one to select directly for cells containing plasmid revertants. Since it would be difficult to isolate spontaneous revertants of a multicopy plasmid by direct selection for temperature-resistant plasmid-containing cells, revertants were induced by mutagenizing plasmid DNA *in vivo* by treating pJN75-containing cells growing at 30° C with N-methyl-N′-nitro-N-nitrosoguanidine. Mutagenized plasmid DNA was then used to transform *E. coli* HB101 and transformants containing plasmids which no longer expressed the ts lethal phenotype were selected by their ability to grow at 37° C in the presence of ampicillin. Plasmids were purified from six temperature-resistant transformants and designated pCM1–pCM6.

The six revertant plasmids were characterized by determining the nucleotide sequence in the region of the *orp* mutation. We found that each of the plasmids still contained the original GC to AT transition at position −23 relative to the RNA I start site. This finding led us to search for secondary mutation sites (see below).

Determination of Plasmid Copy Numbers. In order to confirm our suspicion that the temperature-resistant phenotypes of the pJN75 revertants resulted from suppression of runaway plasmid replication at the restrictive temperature, we examined the copy numbers of pJN75 and two of the revertants before and after a temperature shift from 30° C to 37° C. This experiment is described in Fig. 2. The results of this experiment indicate temperature-induced amplification of the revertant plasmids pCM1 and pCM2, is much lower than that of the ts mutant pJN75. We therefore conclude that the lethal phenotype of pJN75 at the restrictive temperature is related to the plasmid's copy number and that the revertants contain one or more secondary site mutations which suppress the temperature-induced amplification of plasmid DNA.

Mapping the Mutations in the Revertants. The mutations in pCM1–pCM6 were localized to a specific restriction enzyme fragment by subcloning and testing for suppression of the *orp* mutation *in vivo*. The plasmid pJN75 contains two *Hae*II restriction endonuclease recognition sites (positions −186 and +173 relative to the origin of replication) and is cleaved by this enzyme to create a 5,000 bp fragment that contains the *orp* mutation and a smaller 350 bp fragment which contains the origin of DNA replication (Fig. 1). These fragments correspond to the *Hae*II A and E fragments of the parental plasmid NTP1, and are referred to as A and E in this discussion. The plasmids pJN75, pJN70, and each of the revertant plasmids were digested to completion with *Hae*II restriction endonuclease. The A and E fragments were purified from an agarose gel and recombined *in vitro* to create new plasmids. The hybrid plasmids were introduced into *E. coli* HB101 and the viability of the transformants was determined at both 30° C and 37° C. The results of this experiment (Table 2) indicate that the original *orp* mutation falls in fragment A as expected from the DNA sequencing results presented above. The mutations in pCM1–pCM4 that cause suppression of the lethal phenotype at 37° C, however, lie within the 350 bp *Hae*II E fragment. One of the revertant plasmids, pCM5, has lost the

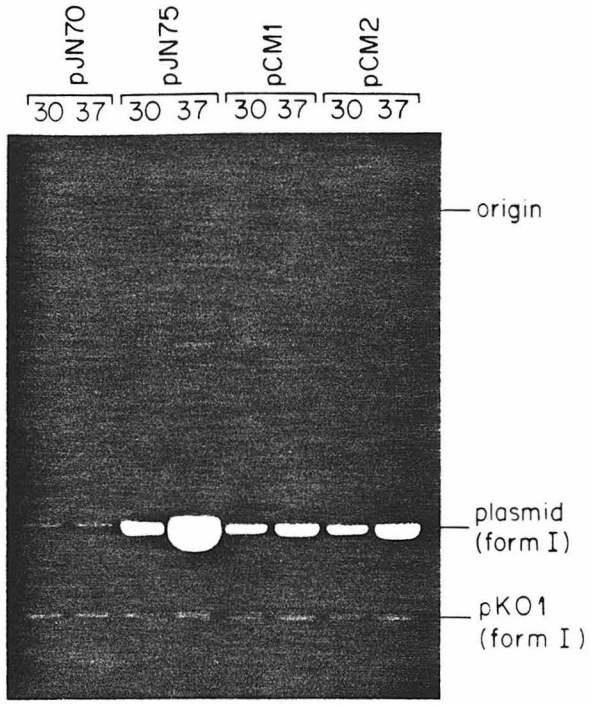

Fig. 2. Effect of temperature-shift on plasmid copy numbers. *E. coli* HB101 cells containing plasmids pJN70, pJN75, pCM1, or pCM2 were grown overnight at 30°C in 5 ml of L broth containing 35 μg/ml ampicillin. The cultures were diluted 1:100 with L broth and allowed to grow at 30° C to an $O.D._{650}$ of 0.15. Half of each culture was then transferred to 37° C and all cultures were incubated for an additional three hours. A portion of the cells in each culture were then combined with an equal number of HB101 cells containing the plasmid pKO1 (from an exponentially growing culture). The pKO1-containing cells were added immediately prior to cell harvest in order to provide a control plasmid for standardizing plasmid DNA recoveries. After harvesting by centrifugation, the cells were lysed and plasmid DNA isolated as described previously (Moser and Campbell 1983). The plasmids were then analyzed by electrophoresis through a 1.0% agarose gel. The lower band in each lane corresponds to the covalently-closed circular form of the pKO1 standard

*Hae*II site at position −187 upstream from the origin. The reversion mutation in the sixth revertant, pCM6, maps within the *Hae*II A fragment (data not shown) and will be described elsewhere.

By determining the DNA sequences of the apropriate *Hae*II E fragments, we have identified five different point mutations in plasmids pCM1–pCM5. These are indicated in Fig. 1 as *sorp1–sorp5*, for suppressor of over-replication. All five mutations are GC to AT transitions which fall within a 128 bp region that begins at the *Hae*II A/E junction at position −186 and extends toward the origin of replication.

Only one of the *sorp* mutations, *sorp4*, occurs within a region of RNA II that exhibits any sequence complementarity with the region surrounding the *orp* mutation. This complementarity is limited to only five nucleotides which re centered around the two mutation sites. Therefore, one obvious explanation for secondary site reversion, the restoration of base pairing within the transcript, cannot be in-

Table 2. Mapping of secondary mutations

HaeII A fragment	HaeII E fragment					
	pJN70	pJN75	pCM1	pCM2	pCM3	pCM4
pJN70	+	+				
pJN75	−	−	+	+	+	+
pCM1	−	−	+			
pCM2	−	−		+		
pCM3	−	−			+	
pCM4	−	−				+

Plasmids pJN70, pJN75 and pCM1 through pCM4 were digested to completion with HaeII restriction endonuclease. The 5.1 kbp A fragment and the 350 bp E fragment from each plasmid were separated by electrophoresis through a 1.0% low-melting temperature agarose gel. Fragments were purified from the gel, and ligated as indicated to construct recombinant plasmids. The ligation products were introduced into *E. coli* HB101 by transformation. Cells were spread onto L plates containing ampicillin (50 μg/ml) and incubated overnight at either 30° C or 37° C. All constructs gave healthy colonies on the plates incubated at 30° C. Those constructs which gave viable transformants at 37° C are indicated in the table by a plus sign (+). A minus sign (−) indicates that the transformants were viable only at 30° C

voked to explain the mechanism by which the *sorp* mutations compensate for the effects of the ts *orp* mutation.

Discussion

Temperature-induced amplification of a conditional high copy-number mutant of plasmid NTP1 is lethal to the host cell. The mutant plasmid, pJN75, contains a single base pair alteration within a 600 bp region of the plasmid that is necessary for plasmid DNA replication. The mutation, designated *orp*, is a GC to AT transition that lies within the coding sequence for the primer precursor, RNA II, but also falls within the promoter for RNA I, the inhibitor of plasmid replication. The *orp* mutation occurs 23 base pairs from the start site of RNA I transcription.

This adds another similarity to the regulation of ColE1 and NTP1 plasmids (see Introduction), since Wong et al. (1982) have described two temperature-sensitive high copy-number mutants of plasmid ColE1 which also contain base pair substitutions within the RNA I promoter region. Our studies, while in a different plasmid, strengthen the conclusion that the ts mutation in ColE1 is due to this alteration, since recombinants prepared in vitro showed the ts mutation maps within a restriction fragment containing the portion of the origin region with this alteration, which had not been previously substantiated. One of the ColE1 mutants, pMM1, contains a single-site alteration at position −21 relative to the RNA I start site. A second mutant, pEW2762, contains point mutations at positions −13 and −23. The −23 mutation in this plasmid is identical to the *orp* mutation in pJN75 in both position and nature (GC to AT transition). It is perhaps noteworthy that a GC base pair occurs at this position in the RNA I promoter of all the related multicopy plasmids (ColE1, pMB1, p15A, NTP1, and CloDF13) (Oka et al. 1979; Sutcliffe 1978; Stuitje et al. 1980; Selzer et al. 1983). This is the only nucleotide pair between the positions −18 and −30 of the RNA I promoter that is conserved in all five plasmids. Promoter-*galK* gene fusion studies of the RNA I promoters from the ts copy-number mutants of ColE1 (Wong et al. 1982) and NTP1 (this work) indicate that the ts mutations do affect transcription but not in a temperature-dependent manner. The *orp* mutation in pJN75 results in a two-fold reduction in the promoter strength as determined from expression of galactokinase activity. This result indicates that the nucleotide sequence between the conserved RNA polymerase recognition sites at positions −10 and −35 may have a role in determining promoter strength.

From the RNA I promoter studies we infer that the conditional high copy-number phenotype of pJN75 does not result from a reduction of RNA I synthesis at the restrictive temperature. Rather, it is likely that the mutation affects RNA II secondary structure. The specific structure that is rendered thermosensitive by the *orp* mutation may be part of the target site for RNA I binding. Alternatively, it may be a structure involved in relaying to the origin structural changes that initiate near the 5′ end of RNA II as the result of RNA I binding and lead to the inhibition of the hybrid formation. The location of the mutation downstream of the region in RNA II that is complementary to RNA I supports the latter idea, but does not rule out the possibility that secondary structure near the *orp* mutation is important for RNA I recognition.

The identification within pJN75 revertants of a number of secondary-site mutations that suppress the over-replication phenotype caused by the *orp* mutation provides new evidence supporting the role of RNA II secondary structure in the regulation of primer formation. The mechanism by which these mutations suppress over-replication is not known. However, one possibility is that they destabilize RNA II structures whose formation during transcription is essential to the formation of the RNA-DNA hybrid near the origin. The *sorp* mutations may compensate for unregulated plasmid replication at the restrictive temperature by reducing the rate or efficiency of hybrid formation.

In support of this, there is other evidence suggesting that secondary structure in the region of the *sorp* mutations is important for replication initiation. Naito and Uchida (1980) have described a cis-dominant replication defective mutant of ColE1 which contains a GC to AT transition mutation at position −160 relative to the ColE1 origin. A revertant that had regained partial function of the ColE1 replicon contains a secondary mutation, a GC to AT transition, at position −187. A potential hairpin structure can be constructed for this region of RNA II which involves pairing of the bases at positions −160 and −187. The DNA sequence coding for this hairpin structure is highly conserved in ColE1, pMB1, NTP1, and CloDF13, but is missing from p15A (Oka et al. 1979; Sutcliffe 1978; Stuitje et al. 1980; Selzer et al. 1983). It is intriguing that two of the mutations, *sorp3* and *sorp5*, which suppress the conditional over-replication phenotype of pJN75, affect base-paired nucleotides within the stem of this structure.

There is a precedent for a regulatory mechanism in which the folding of a nascent transcript into specific secondary structures influences subsequent transcriptional events. In the attenuation model (Yanofsky 1981) proposed for the regulation of transcription from a number of amino acid operons, the formation of a specific hairpin structure during transcription results in early termination. The folding of the RNA into this terminator loop is in turn regulated by the presence and position of a bound ribosome 5′ to the attenuation site. Similarly, the folding of RNA II near

the origin may be regulated by previous folding events near the 5′ end of the transcript. These early folding events appear to be affected by the binding of RNA I (Tomizawa and Itoh 1982). Further characterization of mutants that alter the stabilities of specific RNA II secondary structures should prove helpful in defining the mechanism for the formation of the RNA-DNA hybrid near the origin.

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Chapter 3

Cis-acting mutations that affect rop protein control of plasmid copy number

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Abstract

A number of pMB1 derivatives provide a trans-acting function which can suppress lethal runaway replication of a temperature-sensitive copy-number mutant of NTP1. Deletion analysis indicates that the region of the pMB1 genome which contains the rop gene is required for this suppression. Mutant derivatives of the ts copy-number mutant plasmid whose conditional lethal phenotype is not suppressed in trans by the region encoding the rop gene have been isolated. These rop-insensitive derivatives contain single nucleotide changes within the RNA I coding region.

Introduction

Initiation of replication of ColE1-type plasmids is regulated by two trans-acting negative control elements (Conrad and Campbell 1979; Tomizawa et al. 1981; Twigg and Sherrat 1980; Cesarini et al. 1982; Som and Tomizawa 1983). One of these elements, RNA I, acts as a replicon-specific inhibitor of plasmid replication and is responsible for plasmid incompatibility (Tomizawa et al. 1981; Tomizawa and Itoh 1981; Moser and Campbell 1983). Purified RNA I inhibits the processing of a second plasmid transcript, RNA II, in vitro. This processing is necessary to form the primer for initiation of DNA synthesis. The target of RNA I inhibition is believed to lie within a region of RNA II that is complementary to RNA I (Cesarini 1981; Lacatena and Cesarini 1981; Tomizawa and Itoh 1981). Both RNA I and its complementary region on RNA II may adopt a secondary structure with three stem-loop structures. Genetic studies indicate that the single-strand loops are involved in the inhibitory activity of RNA I, which occurs by base-pairing with the complementary sequence of RNA II (Lacatena and Cesarini 1981; Tomizawa and Itoh 1981). The hybridization of the two transcripts prevents the formation of an RNA-DNA hybrid structure between RNA II and its template near the replication origin (Tomizawa et al. 1981). The RNA-DNA hybrid is a substrate for ribonuclease H which cleaves the transcript at the origin of replication to create the 3'-OH end of the primer. (Itoh and Tomizawa 1980).

The second element in the copy control system is the product of a gene located 500 bp downstream from the replication origin of ColE1 and pMB1 (Twigg and Sherrat 1980; Cesarini et al. 1982; Som and Tomizawa 1983). Twigg and Sherrat (1980) observed that deletions of this region in ColE1 and pMB1 derivatives caused an increase in the plasmid copy numbers. They also showed that the elevated copy numbers could be reduced to wild-type levels by providing the deleted function in trans from a second compatible plasmid in the cell. Cesarini et al. (1982) reported that in vivo expression of the lacZ gene directed by the primer promoter was reduced in the presence of a plasmid carrying this "repressor." They proposed that the repressor is a 63 amino acid polypeptide that is conserved in both ColE1 and pMB1. They speculated that this repressor, which they designated rop (for repressor of primer), acted independently of RNA I to regulate plasmid replication by limiting transcription initiation of RNA II, the primer precursor.

We have observed that a temperature-sensitive runaway replication mutant plasmid fails to express its conditional lethal phenotype in the presence of certain compatible plasmids. Genetic evidence suggests that this suppression is mediated by the rop gene encoded on the second plasmid. We have taken advantage of this activity of rop to select mutant derivatives of the temperature-sensitive plasmid that are insensitive to rop suppression. DNA sequencing of a number of these rop-insensitive derivatives gives

new information about the mechanism of rop-mediated regulation of plasmid replication.

Materials and Methods

Bacterial strains and plasmids

All of the transformation and plasmid studies were carried out in Escherichia coli HB101 pro gal hsdR hsdM recA1. The plasmid pJN75 is a 5.4 kb ampicillin-resistant derivative of NTP1 (Moser et al. 1983; Grindley et al. 1978). pJN75 exhibits a temperature-sensitive mutant copy number phenotype. The presence of the plasmid is lethal to the host cell at the restrictive temperature 37°C. The lethality is a result of runaway plasmid replication. A single nucleotide difference from the wildtype NTP1 sequence within the region required for replication appears to be responsible for the ts mutant phenotype (Moser et al. 1983). This mutation is a GC to AT transition located 398 bp upstream of the origin of DNA synthesis. Plasmid pMB9 is a tetracycline-resistant derivative of pMB1. Other plasmids are described in Table 1.

Isolation of pJN75 mutants whose ts runaway replication phenotype is not suppressed by the rop gene product

A culture of E. coli HB101 containing pJN75 was grown at 30°C to an A_{590} of 0.3 in 5 ml of L broth. Chloramphenicol (100 µg/ml) and N- methyl-N'-nitro-N-nitrosoguanidine (20 µg/ml) were added and incubation was continued overnight. Plasmid DNA was isolated from the cells and introduced into E. coli HB101 containing pMB9 by the transformation procedure of Dagert and Ehrlich (1979). Cells were

spread onto L plates containing tetracycline (15 µg/ml) and a high concentration of ampicillin (4 mg/ml) and incubated at 34°C overnight. Under these conditions cells containing pJN75, which is at high copy number, survive. Cells containing both pJN75 and pMB9, however, do not form colonies under these conditions, presumably because rop suppression of pJN75 replication reduces the copy number of pJN75 to a level below what is needed to confer resistance to 4 mg/ml ampicillin. Mutants of pJN75 that are insensitive to the rop suppression are able to grow in the presence of pMB9 at 34°C on 4 mg/ml ampicillin. Several hundred colonies grew up overnight from cells transformed with the mutagenized DNA whereas no colonies were obtained when nonmutagenized DNA was used. Colonies were then tested for expression of the ts lethal phenotype by replica plating colonies onto two sets of plates containing tetracycline (15 µg/ml) and low ampicillin (50 µg/ml) and growing one set at 30°C and the other set at 42°C overnight. Approximately 25% of the colonies grew at 30°C but did not grow at the higher temperature. Plasmid DNA was purified from eight temperature-sensitive colonies.

DNA sequence analysis

The nucleotide sequence surrounding the replication origin of pJN75 has been reported previously (Moser et al. 1983; Grindley and Nakada 1981). The sequence of the origin region of eight pJN75 mutants was determined by the chemical degradation method of Maxam and Gilbert (1980). Approximately 800 nucleotides were sequenced

over a region extending from 700 nucleotides upstream of the origin of DNA synthesis to 100 nucleotides downstream of the origin.

Results

Suppression of pJN75 runaway replication by the rop gene product

Plasmid pJN75 is a temperature-sensitive copy-number mutant derivative of plasmid NTP1. (Grindley et al. 1978; Moser et al. 1983). [Although isolated independently, NTP1 is apparently identical to the plasmid RSF1030 (Conrad, Wold, and Campbell, 1979; Grindley and Nakada, 1981; Som and Tomizawa, 1982)]. Bacteria containing pJN75 grow normally at the permissive temperature (30°C) but cease growth and die one to two generations after a shift to the nonpermissive temperature (37°C). Loss of viability appears to be a consequence of runaway replication which results in an increase in plasmid synthesis from about 8% of chromosomal DNA synthesis to 150% in the first 10 min after the temperature shift (Grindley et al. 1978).

We have previously identified the mutation in pJN75 responsible for the ts mutant replication phenotype (Moser et al. 1983). This mutation is a single base pair change, a GC to AT transition, located 398 bp upstream of the origin of DNA synthesis. The temperature-induced over-replication phenotype is most likely the consequence of thermolability of a secondary structure within the primer precursor which is normally essential for regulation, though this has not been shown conclusively (Moser et al. 1983; Wong et al. 1982). The inability of this structure to form at the restrictive temperature may render replication of the plasmid insensitive to

normal regulation through RNA I inhibition.

We have observed that runaway replication of pJN75 at the nonpermissive temperature is suppressed in the presence of certain compatible plasmids. This suppression was first observed in double transformants of pJN75 and pMB9, a tetracycline-resistant derivative of the plasmid pMB1. The double transformants grew normally at 42°C as well as at 30°C in the presence of both ampicillin and tetracycline. Plasmid DNA levels were analyzed from lysates of cells grown in liquid cultures before and several hours after a shift to the restrictive temperature. Although this analysis was somewhat complicated by the similarity in molecular weights of pMB9 and pJN75 we were still able to observe that with pMB9 present in the cell, there was very little temperature-induced amplification of pJN75 DNA (data not shown). Suppression of the conditional lethal phenotype of pJN75 was also observed with the pMB1 derivatives, pBR322 and pBR325 (Table 1). The electrophoretogram shown in Figure 1 illustrates the effect of a plasmid derived from pBR325 on the temperature-induced amplification of pJN75 DNA.

NTP1 is compatible with pMB1-derived plasmids, i.e., NTP1 and pMB1 derivatives can stably coexist in the same cell. This is apparently a consequence of the fact that the RNA I species encoded by NTP1 is not identical to the pMB1 RNA I species. In vitro experiments described by Tomizawa and Itoh (1982) indicate that purified NTP1 (RSF1030) RNA I does not inhibit primer formation on a ColE1 template and that ColE1 RNA I does not inhibit NTP1 primer

formation. Since there is apparently no cross-inhibition of plasmid replication by the two wild-type RNA I species, we speculated that a negative control element other than RNA I was responsible for the suppression of pJN75 replication by the pMB1 derivatives. The inability of the plasmid pDM254 (Moser and Campbell 1983), which contains multiple pMB1 RNA I genes, to suppress pJN75 runaway replication supports this notion (Table 1). Since the pMB1 derivatives which provide suppressor activity also encode the rop gene described by Cesarini et al. (1982), the rop gene product seemed a likely candidate for the trans-acting suppressor. Therefore, we looked at the effect of co-cultivation of pJN75 with several pMB1-derived plasmids that contained deletions of all or part of the rop gene (Table 1). No suppression was observed with the pBR322 derivatives pAT153, pKO1, or pUC8. Plasmid pAT153 differs from pBR322 by a deletion of the 622 bp HaeII restriction fragment which encodes the entire rop gene. Plasmids pKO1 and pUC8 contain a deletion of the 3' end of the rop gene. These results imply that the rop gene product is responsible for the suppression of the runaway replication phenotype of pJN75.

Isolation of mutant derivatives of pJN75 insensitive to rop suppression.

These observations suggested a way to identify the site where rop interacts in suppressing pJN75 runaway replication, namely, by isolating mutants of pJN75 that are insensitive to rop-mediated

suppression. The rationale for selecting such mutants depends on the idea that at temperatures intermediate between permissive (30°C) and non-permissive (37°C), the copy number of the mutant plasmid should be elevated but not lethal to the cells. To test this idea, cells carrying pJN70 or pJN75 were plated at 34°C on L agar plates containing a high concentration of ampicillin (4 mg/ml). As expected, cells carrying pJN75 were able to grow because the copy number was high, while cells carrying the wild-type plasmid did not produce enough β-lactamase to survive. Furthermore, cells carrying pJN75 plus pMB9 did not form colonies under these conditions, presumably because rop-mediated suppresssion of pJN75 replication reduces the copy number to a level below what is required to confer resistance to 4 mg/ml ampicillin. In order to isolate a rop-insensitive plasmid, mutagenized pJN75 DNA was used to transform cells that contained pMB9 at 34°C. Only pJN75 derivatives insensitive to replication inhibition by rop function were expected to grow. Several hundred such colonies were isolated and shown to retain the ts lethal phenotype at 42°C, indicating that the original mutation in pJN75 was still present. Eight of the colonies able to grow at 34°C but not at 42°C, were selected for further characterization. Analysis of the DNA content of these cells revealed that both plasmids (pMB9 and the pJN75 derivative) were present as monomers, suggesting the phenotype was due to a mutation of the pJN75 genome. The plasmids exhibiting the non-suppressed

phenotype in the presence of rop were designated pJN75nsr for nonsuppressible by rop.

Identification of the nsr mutations

The nucleotide sequence surrounding the replication origin of the nsr derivatives of pJN75 was determined following the procedure of Maxam and Gilbert (1980). Each of the mutant plasmids was found to contain the original pJN75 alteration and a single additional base pair change within the 800 bp region extending from 100 bp downstream of the origin of DNA synthesis to 700 bp upstream of the origin. Four of the mutants contained a GC to AT transition at position -439 (nsr1). [Position +1 is defined as the site of incorporation of the first deoxynucleotide during the initiation of plasmid DNA replication.] The other four mutants contained a GC to AT transition 10 bp away at position -449 as shown in Figure 2. Both mutations cause disruption of base-pairing within the stem of loop-structure III in the primer transcript and loop- structure III' of RNA I (Figure 2). This result is consistent with the galK fusion studies of Som and Tomizawa (1983), that indicate that sequences important for rop function do not fall in the promoter for RNA II, but instead lie further downstream. The mutations we have identified define this region to be within the coding sequence for both RNA I and RNA II, near the 5' terminus of RNA I.

Discussion

In this paper, we have shown that the rop gene provides a trans-acting function that can suppress runaway replication of the NTP1 ts copy-number mutant, pJN75. This conclusion is based on the observation that plasmids with deletions of part or all of the rop gene lack the suppressor activity. This interpretation is also supported by previous reports that the rop gene acts in trans to lower plasmid copy number (Twigg and Sherrat 1980; Som and Tomizawa 1983).

We have used the ability of the rop gene product to suppress the ts lethal phenotype of the runaway replication mutant, pJN75, to further investigate the general mechanism by which rop modulates plasmid copy number. Although the molecular details of this inhibition are not yet understood, one model has been that the rop gene product may be inhibiting transcription of RNA II and therefore controlling replication by limiting the amount of precursor RNA available for primer formation. This idea is supported by the observations of Cesareni et al. (1982) and Som and Tomizawa (1983) that the rop gene product inhibits production of β-galactosidase or galactokinase when the lacZ or galK genes are fused to the primer downstream of the primer promoter. In order to investigate how such inhibition might occur, we looked for mutants resistant to inhibition by the rop gene product as described above. We have identified mutants of pJN75 which express the lethal runaway

replication phenotype at 42°C even in the presence of the rop gene encoded on a second plasmid in the cell. Determination of the nucleotide sequence of eight mutant plasmids revealed mutations at only two points, both within the RNA I coding region. Although located 10 nucleotides apart, the two mutations disrupt adjacent base pairs within a stem-loop structure present in both RNA I and RNA II. This finding, along with that of Som and Tomizawa (1983) that a region within the coding sequence of RNA II, rather than in the primer promoter, was necessary for inhibition of galactokinase production in the fusion studies, suggests that the rop protein does not act by binding to an operator site near or within the promoter and competing with RNA polymerase binding. This is also consistent with the fact that the rop protein from pMB1 can inhibit replication of plasmids such as NTP1 which have virtually no sequence homology to pMB1 in the DNA immediately 5' to the primer RNA, in the primer promoter.

In light of the finding by Som and Tomizawa (1983) that the rop protein enhances hybrid formation between RNA I and its complementary region of RNA II, it is consistent that the selection for mutants of the rop interaction site yielded only plasmids with mutations in the region of RNA I and RNA II overlap. It is possible that the rop protein may recognize one or more of the stem-loop structures on either RNA I or RNA II as the binding site. Binding of the rop protein may then favor the interaction of RNA I and its target region of RNA II. It is perhaps worth noting that the two

nucleotide pairs affected by the mutations nsr1 and nsr2 are conserved in the RNA I species from ColE1, p15A, RSF1030 and CloDF13 (Selzer et al. 1983). In addition, on the same stem structure immediately adjacent to these paired bases is an unpaired nucleotide which is also conserved in these plasmids. It is imaginable that this "spur" on the stem could be a part of a recognition site for the binding of a small protein.

Identification of a rop-nucleic acid interaction site by genetic analysis is complicated by the fact that rop target mutations may also alter the structure of RNA I or RNA II such that the inherent ability of these RNAs to interact with each other is diminished. An altered copy-number phenotype associated with a mutation in the RNA I coding region can be a consequence of either a defective RNA I inhibitor, an altered RNA I target site within the primer transcript, or the inability of the rop protein to interact with either of the mutant transcripts. It is quite likely that all of these functions could be affected by a single point mutation. The mutation nsr2 that we have identified affects a nuclelotide at the same position in pJN75 RNA I as the svir19 mutation of pMB1 described by Lacatena and Cesareni (1981). The latter mutation was obtained using a "phasmid" selection designed to isolate mutants exhibiting reduced sensitivity to the wild-type replication inhibitor, RNA I. When released from the lambda chromosome the mutant plasmid containing the svir19 mutation was lethal for the host cell. We have also found that both nsr mutants are lethal to

the host cell in the absence of pMB9 or other compatible rop$^+$ plasmid (data not shown). This lethality is independent of temperature and is assumed to result from uncontrolled or runaway plasmid replication, although this has not been demonstrated either for sviri9 or for the nsr mutants. These results are consistent with the idea that the mutations nsr1, and nsr2 in NTP1, and possibly sviri9 in pMB1, affect both the RNA I-RNA II interaction and the binding of the rop protein, and support the finding that rop enhances the hybridization of the two RNAs.

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Table 1. Trans-suppression of pJN75 runaway replication by various compatible plasmids

Plasmid	Incompatibility class	Presence of *rop* gene	Suppression of ts lethal phenotype of pJN75-containing cells
pMB9	pMB1	+	+
pBR322	"	+	+
pBR325	"	+	+
pAT153	"	-	-
pK01	"	-	-
pUC8	"	-	-
pACYC184	p15A	(-)?	-
pDM254	p15A,pMB1	(-)?	-

E. coli HB101 cells containing pJN75 were transformed with the indicated plasmids following the transformation procedure of Dagert and Ehrlich (1979). Cells were spread onto L plates containing ampicillin (50 ug/ml) which were then incubated overnight at 42° C. The plasmids which produced temperature-resistant colonies with a high transformation effiency (>10^6/ug plasmid DNA) are indicated by a plus sign (+). A minus sign (-) indicates those plasmids which did not give temperature-resistant colonies. Plasmids pMB9, pBR322, and pBR325 are all pMB1 derivatives which contain the region of the plasmid genome encoding the *rop* gene. The plasmid pAT153 (Twigg and Sherrat 1980) is identical to pBR322 except for a deletion of a 622 bp Hae II restriction fragment that contains the entire *rop* gene (Cesareni et al. 1982). Plasmids pK01 (McKenney et al. 1981) and pUC8 (Viera and Messing 1982) are also derived from pBR322 and contain only the portion of the *rop* gene which codes for the last nine amino acids of the 63 amino acid polypeptide. The plasmid

pACYC184 contains the replication origin from p15A (Chang and Cohen 1978). There is no evidence of a *rop* gene encoded on pACYC184 or p15A. Plasmid pDM254 (Moser and Campbell 1983) is a pACYC184 derivative with four copies of the pMB1- RNA I gene cloned in tandem at the *Bam*HI site.

Figure Legends

Figure 1. Suppression of temperature-induced amplification of pJN75 DNA by a pMB1 derivative carrying the *rop* gene. HB101 cells carrying either pJN75 (Ap^r) or pJN75 and the plasmid pAC16 (Tc^r, Cm^r) were grown in L broth containing the appropriate antibiotics at 30°C to an A_{590} of 0.15. Half of each culture was then transferred to 37°C and all cultures were incubated for an additional three hours. A portion of each culture was then mixed with a portion of another cell culture containing an equal number of HB101 cells carrying the plasmid pKO1 (McKenney et al. 1981). The pKO1-containg cells were added immediately prior to cell harvest in order to provide a control plasmid for standardizing plasmid DNA recoveries. After harvesting by centrifugation, the cells were lysed and total plasmid DNA isolated as described previously (Moser and Campbell 1983). The covalently-closed circular plasmids were analyzed by electrophoresis through a 1.0% agarose gel. The plasmid pAC16 (Garfinkel et al. 1982) is a pBR325 derivative which contains a 700 bp rat actin cDNA insert cloned into the *Pst* I site and therefore does not confer ampicillin resistance to its host.

Figure 2. RNA I transcript from plasmid pJN75. Mutations *nsr*1 and *nsr*2 are those identified within the mutant pJN75 derivatives which were selected by their temperature-sensitive phenotypes in the presence of pMB9. The RNA I secondary structure shown is that

proposed by Som and Tomizawa (1982) based on the secondary structure for ColE1 RNA I proposed by Morita and Oka (1979).

Figure 1

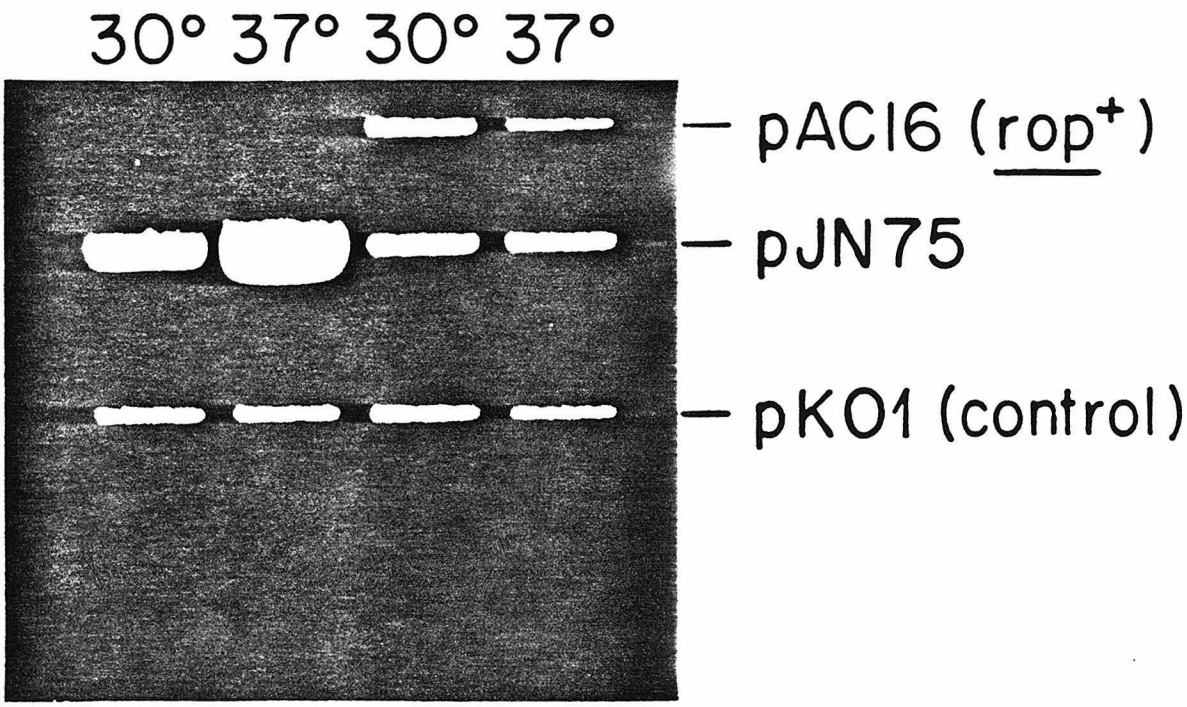
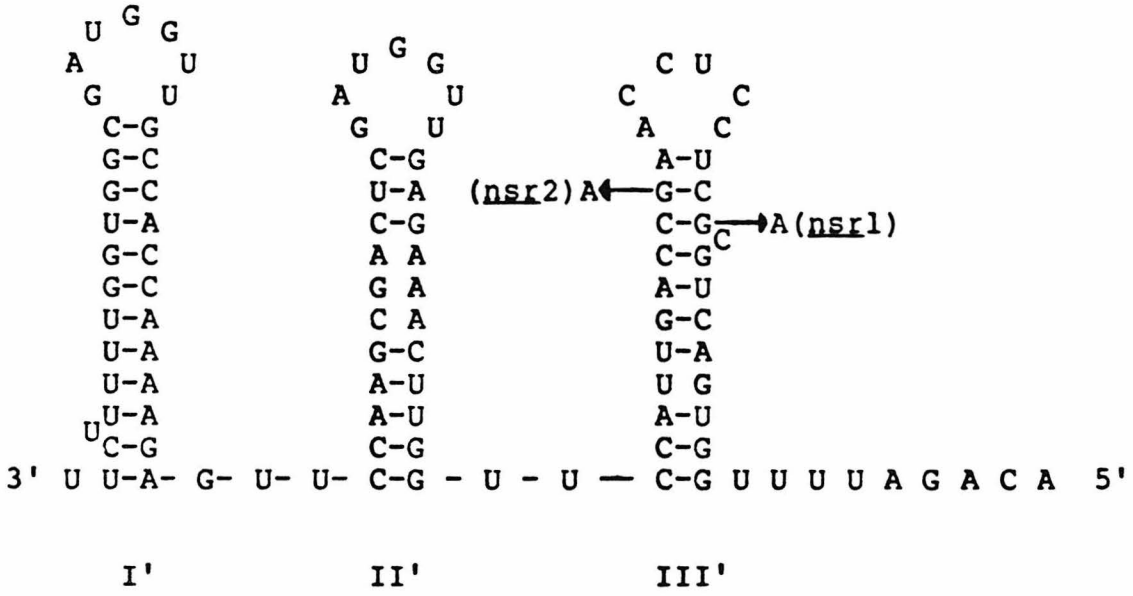


Figure 2



Chapter 4

Identification of the Target Region for Inhibition of Plasmid DNA Replication by RNA I

Abstract

Because the inhibitor of plasmid replication and its site of inhibition are encoded by the same region of the pMB1 genome, mutations which alter the target also alter the inhibitor. A special pMB1 derivative has been constructed in which the gene coding for the active replication inhibitor, RNA I, does not overlap with the region that codes for the 5' end of RNA II, the putative target of RNA I inhibition. Dominant high copy-number mutants of the recombinant plasmid pDM247 contain mutations that alter only the target site. Six different point mutations have been identified, all of which affect nucleotides within one of the single-stranded loops of the three stem-loop structures that can form near the 5' end of RNA II. The location of these mutations suggest that the central loop structure of RNA II has a primary role in the initial interaction with RNA I that results in the inhibition of plasmid DNA replication.

Introduction

All of the information necessary for maintenance of ColE1 as a plasmid lies within a 580 base pair fragment derived from the origin region (Backman et al. 1978; Oka et al., 1979;). Two RNA elements are specified by this region. One of these elements, RNA I, is the trans-acting negative regulator of ColE1 replication (Conrad and Campbell, 1979; Muesing et al., 1981; Tomizawa et al. 1981). RNA I is 108 nucleotides long and is transcribed in the direction opposite to DNA replication from a promoter located 450 bp upstream of the plasmid origin (Morita and Oka, 1979). Transcription of the second element, RNA II, initiates near the termination site of RNA I proceeds in the opposite direction to RNA I synthesis and terminates downstream of the origin (Itoh and Tomizawa, 1980). Consequently, the nucleotide sequence of RNA I is complementary to the nucleotide sequence at the 5'-terminus of RNA II.

Approximately one-third of the RNA II molecules transcribed in vitro form a stable RNA-DNA hybrid with the template DNA near the plasmid origin (Itoh and Tomizawa, 1980). This RNA-DNA hybrid is a substrate for RNase H which cleaves the RNA close to the origin leaving a structure where the transcript is held to the template by only a small number of base pairs. The 3'-OH end of this structure can be extended by DNA polymerase I to initiate DNA synthesis.

The association of RNA II transcription and the formation of the RNA-DNA hybrid at the origin is referred to as "coupling" (Tomizawa and Itoh, 1982). Purified RNA I prevents primer formation

in vitro by inhibiting the coupling process (Tomizawa et al. 1981). This inhibition by RNA I has been shown to be incompatibility-group specific (Tomizawa and Itoh, 1981). The region that determines the specificity of response to RNA I maps more than 300 bp upstream of the origin of replication (Tomizawa et al., 1981).

It has been suggested that RNA I interacts with nascent RNA II during transcription in such a way as to induce a change of secondary structure in RNA II that prevents the formation of the RNA-DNA hybrid at the origin (Tomizawa and Itoh, 1981; Tomizawa and Itoh, 1982). Because the nucleotide sequence of RNA I is complementary to the 5' end of RNA II the transcripts are capable of hybridizing to each other. Both RNA I and the complementary region of RNA II have extensive internal sequence symmetry and can form three prominent stem and loop structures (Morita and Oka, 1979). The interaction between these RNAs may be initiated by base-pairing of the single-stranded loops of these structures. In vitro transcription studies show that single base changes in any of the three structures affect both the ability of RNA I to inhibit primer formation and the sensitivity of primer formation to inhibition by RNA I (Tomizawa and Itoh, 1981). These base changes also alter the rate of association between RNA I and RNA II.

Lacatena and Cesareni (1981) have described a "phasmid" system which they used to obtain mutants in the target of the negative regulator of pMB1 replication. The identification of single base-pair changes in the region of RNA I-RNA II overlap prompted the

authors to propose that the target of the replication inhibitor coincides with the DNA region that codes for the inhibitor itself. The locations of the base substitutions point to the primary importance of the central loop structures in the interaction between RNA I and RNA II.

An important consequence of a unique plasmid region encoding both the replication inhibitor, and the site of its inhibition is that a single base pair substitution within this region can alter the specificity of RNA I inhibition without impairing the plasmid's ability to to regulate its own copy number (Lacatena and Cesareni, 1981). A mutation in the target of RNA I inhibition creates a complementary change within RNA I so that the potential for base pairing between the RNAs remains unchanged. While this property allows for the evolution of plasmids of different compatibility groups, it hampers mutational studies designed to identify the nucleotides that are involved in the initial RNA-RNA interaction.

In this paper, we describe the isolation of high copy-number mutants of a unique recombinant plasmid in which the region coding for the replication inhibitor does not overlap the RNA II coding region. The plasmid pDM247 was constructed by the insertion of two BamHl fragments, each coding for transcription of the pMBl RNA I species, into the unique BamHl site of the copy-number mutant pFHl18 (Moser and Campbell, 1983). A 16 bp insertion mutation in the pFHl18 RNA I coding sequence impairs the ability of this transcript to regulate replication. The copy number of pDM247, which is 60

times lower than that of pFH118, is controlled by the wild-type RNA I transcribed from the BamH1 inserts. The high copy mutants of pDM247 that we have isolated contain mutations exclusively in the target of RNA I inhibition. These mutations implicate a critical role of the central loop in RNA II in the mechanism of plasmid copy-number control.

Materials and Methods

Bacterial strains and plasmids

All plasmid studies were carried out in *Escherichia coli* HB101 *pro gal hsdR hsdM recA1*.

Plasmids pDM246 and pDM247 have been previously described (Moser and Campbell, 1983). Both plasmids are derived from the high copy-number mutant pFH118. pFH118 was constructed by linker insertion mutagenesis of the pMB1-derivative RSF1050 (Heffron et al., 1978). This mutagenesis procedure creates a new *Eco*R1 endonuclease recognition site at the site of insertion. In pFH118 this site is located approximately 500 bp upstream of the origin of replication within the RNA I coding region (Conrad and Campbell, 1979). The plasmids pDM246 and pDM247 contain one and two copies, respectively, of a 256 bp restriction fragment from RSF1050, inserted at the unique BamH1 restriction site of pFH118. This fragment encodes the wild-type RNA I gene and its promoter but lacks the promoter for RNA II. The copy numbers of plasmids pFH118, RSF1050, pDM246 and pDM247 are 720, 60, 24 and 12, respectively (Moser and Campbell, 1983).

Isolation of cop^- mutants of pDM247

Thirty cultures of *E. coli* HB101 carrying the plasmid pDM247 were grown overnight at 37°C in 5 ml of L broth containing ampicillin (50 μg/ml). The cells from 1.0 ml of each culture were collected by centrifugation, resuspended in 0.1 ml of L broth and spread onto a single L plate containing 0.2% glucose, ampicillin

(0.6 mg/ml) and methicillin (4 mg/ml). Following overnight incubation at 37°C, 30-60 colonies appeared on each of the plates. A single colony was taken from each plate for further characterization. Nineteen of the thirty isolates contained elevated levels of plasmid DNA. HB101 cells transformed with plasmid DNA isolated from these colonies were able to grow on plates containing a high concentration of ampicillin (0.6 mg/ml) whereas similar cells carrying pDM247 did not grow at this drug level indicating that the elevated copy numbers of these plasmids are caused by plasmid mutations rather than mutations within the host chromosome.

DNA sequence analysis

The nucleotide sequence of the origin region between positions -585 and -313 was determined for each of the high copy-number mutants. (The site of incorporation of the first deoxynucleotide in plasmid DNA synthesis is defined as position +1). Plasmids were first digested with EcoRI and the resulting fragments were end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase or by filling in the 3'-ends with [α-^{32}P]dATP using DNA polymerase I (Klenow fragment). The labelled fragments were then cleaved with HinfI and FnuDII restriction endonucleases and the resulting fragments were separated by electrophoresis through a polyacrylamide gel. Two of the radioactively-labelled fragments (220 bp and 60 bp) were eluted

from gel slices and sequenced by the method of Maxam and Gilbert (1980).

Results

The mutant high copy-number plasmid pFH118 has been described previously (Moser and Campbell, 1983). A 16 bp insertion within the RNA I coding region of this plasmid gives rise to a 12-fold increase in plasmid copy number over that of the homologous wild-type plasmid RSF1050. We found that the mutant copy-number phenotype of pFH118 was suppressed when a restriction fragment which encodes the wild-type RNA I from RSF1050 was inserted into a nonessential region of the plasmid genome. The copy number of this recombinant plasmid, pDM246, is 30-fold lower than that of pFH118. We concluded from this result that the 16-additional nucleotides in the RNA I transcript from pFH118 diminishes the ability of the transcript to inhibit initiation of plasmid replication. In addition, the complementation of the mutant phenotype by the wild-type RNA I species implies that the RNA I interaction site on the primer precursor, RNA II, is not directly affected by the insertion mutation.

Since the gene coding for the wild-type replication inhibitor in pDM246 does not overlap the DNA sequence encoding the inhibitor target site, this plasmid is well-suited for mutational studies of the copy control regions. In cop$^-$ mutants of pDM246 target mutations can be readily distinguished from mutations which affect RNA I function. Since we were primarily interested in isolating target mutants, instead of pDM246 we used the closely related plasmid pDM247 for our mutational studies. This plasmid differs

from pDM246 in that it contains two copies of the cloned wild-type RNA I gene (Moser and Campbell, 1983). Since mutations affecting RNA I function are recessive, the probability of isolating a cop$^-$ mutant of pDM247 with mutations in both RNA I genes is extremely low. Target mutations, on the other hand, are dominant mutations, so that essentially all of the pDM247 cop$^-$ mutants should contain alterations within the RNA II coding region.

Cells containing cop$^-$ mutant plasmids can be selected directly on plates containing elevated levels of ampicillin. However, because of the problem of crossfeeding which arises from the depletion of ampicillin in the medium surrounding colonies secreting β-lactamase, the selection is only stringent when the density of plated cells is relatively low. In order to increase the number of cells which could be spread per plate, we adapted the methicillin selection procedure described by Wong et al. (1982). This selection exploits the fact that plasmid-encoded β-lactamase has a K_m for the antibiotic methicillin which is 1/50 that of ampicillin. Although methicillin alone is not toxic to cells, it can be added to ampicillin selection plates to potentiate the effect of the antibiotic.

We found that when approximately 10^8 pDM247-containing cells from an overnight culture were spread onto a plate containing both methicillin (4 mg/ml)and ampicillin (0.6 mg/ml), approximately 30-60 colonies appeared after an overnight incubation at 37°C. To avoid isolating resistant colonies that were siblings, thirty overnight

cultures were each plated onto single methicillin-ampicillin plates. Only one colony was then selected from each plate for further characterization. Nineteen of the thirty colonies were found to contain elevated levels of plasmid DNA as detected by gel electrophoresis of plasmid DNA obtained from cell lysates. HB101 cells that were transformed with these plasmid DNAs also exhibited resistance to a high level of ampicillin (0.6 mg/ml), indicating that plasmid mutations were responsible for the high copy phenotypes.

Anticipating that the cop$^-$ plasmids would contain nucleotide alterations upstream of the origin within the plasmid region encoding the 5'-end of RNA II, the DNA sequence of each of the mutant plasmids was examined between positions -585 and -313. A single point mutation was found within a seven base-pair region in each of the nineteen mutants. This region encodes the central loop of the three stem-loop structures near the 5'-end of RNA II. Six different mutations affecting four of the seven nucleotides that comprise the central loop have been identified. The location of the mutations, shown in figure 1, is consistent with the findings of Lacatena and Cesareni (1981) that the central loop of RNA II has a primary role in defining the target of replication inhibition. The base substitutions described here reduce the sequence complementarity of the RNA II central loop and the central loop of the wild-type RNA I. While the mutations in which thymine or adenine is substituted for one of the two cytosines at the center of

the loop structure in RNA II would be expected to significantly reduce the base-pairing potential of the two RNAs (by the elimination of a G-C bond), the mutations affecting the bases closest to the stem of this structure may prevent target recognition by extending the number of paired bases within the stem and promoting closure of the central loop. The three nucleotides for which no mutations were identified could also be involved in the initial base pairing interaction with RNA I. Since substitutions at these sites would result in the loss of a relatively weak A-U base pair, plasmid copy control may not be as severely affected as with the mutants we have isolated. Consequently, cells containing these plasmids may not be resistant to the level of ampicillin used in our selection procedure.

Discussion

By selecting for plasmid-containing cells that exhibit an increased resistance to ampicillin, we have isolated a number of spontaneous mutants of the plasmid pDM247 which have elevated copy numbers. Because mutations affecting RNA I are recessive, and because pDM247 has two copies of the wild-type RNA I gene, the selection favors the isolation of dominant mutations in the target of RNA I inhibition. Of the nineteen mutants so far examined, all nineteen have been found to contain single base pair changes in the RNA II coding region more than 450 bp upstream from the origin of replication. Six different mutations have been identified, all of which alter nucleotides within the central loop structure in the region of RNA II that is complementary to RNA I. Each of the mutations affects potential base pairing of this loop with the central loop of the wild-type RNA I although loop closure can be invoked as an alternative explanation for the phenotype of two of the mutants.

Lacatena and Cesareni (1981), who used a "phasmid" system to select for mutations in the target of the pMB1 replication inhibitor, also found that the central loop in RNA II plays a primary role in the initial interaction with RNA I that leads to inhibition of primer formation. The phasmid selection stems from the authors' observation that a lambda-pMB1 hybrid phage, or "phasmid", was capable of growth on a homo-immune lysogen, presumably because replication by the pMB1 replicon results in

titration of the lambda repressor. However, the presence in the lysogenic strain of a resident pMB1-derived plasmid prevents virulence. This effect is interpreted to be a consequence of the inhibition of replication of the incoming phasmid by RNA I present in the cell. The authors reasoned that mutant phasmids which were capable of growth in the presence of a resident pMB1 plasmid should contain mutations in the target of the pMB1 replication inhibitor. A number of inhibition-insensitive mutants (svir mutants) were isolated and six were extensively characterized. All six svir mutants had single base pair substitutions within the region of RNA I-RNA II overlap. Five of these changes affected nucleotides in the central loop of each transcript. The sixth mutation affected base pairing within the stem of loop structure III in RNA II.

Although the mutations described here and the svir mutations described by Lacatena and Cesareni (1981) lead to a similar conclusion, i.e., that base pairing between the central loop of RNA I and the complementary structure on the nascent primer precursor is a critical step for inhibition of plasmid replication, there is a significant difference in the criteria used for the selection of target mutants. Because the phasmid system selects for mutants that are insensitive to the replication inhibitor synthesized by the resident pMB1 plasmid, it is really a selection for mutants with altered incompatibility. We describe a direct selection for plasmid mutants which exhibit altered copy-number control. The fact that similar mutations have been obtained by

different methods of selection lends additional support to the premise that replication inhibition by RNA I is responsible for both plasmid copy control and the related phenomenom of plasmid incompatibility.

Our previous studies with the high copy-number mutant pFH118 indicate that loop structure I' of RNA I (see figure 1) is important for the inhibitory activity of the molecule, since the disruption of base pairing in the stem of this structure by a small insertion seriously impairs the ability of the transcript to inhibit replication initiation (Moser and Campbell, 1983). In contrast this same plasmid mutation does not significantly affect the plasmid's abilility to respond to inhibition by the wild-type RNA I. This observation, along with the results described here, suggests that the interaction of RNA I and RNA II that leads to inhibition of primer formation involves at least stem-loop structures I' and II' of RNA I and stucture II of RNA II. Structure I of RNA II is probably not essential for the interaction. Less is known about the roles of structures III and III'. Tomizawa and Itoh (1981) have isolated incompatibility mutants of ColE1 which contain single base-pair substitutions that are located in each of the three loop regions. It is not altogether clear which RNA functions are altered by these changes.

A more extensive analysis of the RNA I-RNA II interaction will probably require the construction of plasmid mutants with specific nucleotide alterations using in vitro mutagenesis techniques.

Because the RNA I and RNA II molecules are encoded in different plasmid regions, the plasmids pDM246 and pDM247 would be well-suited for this type of study.

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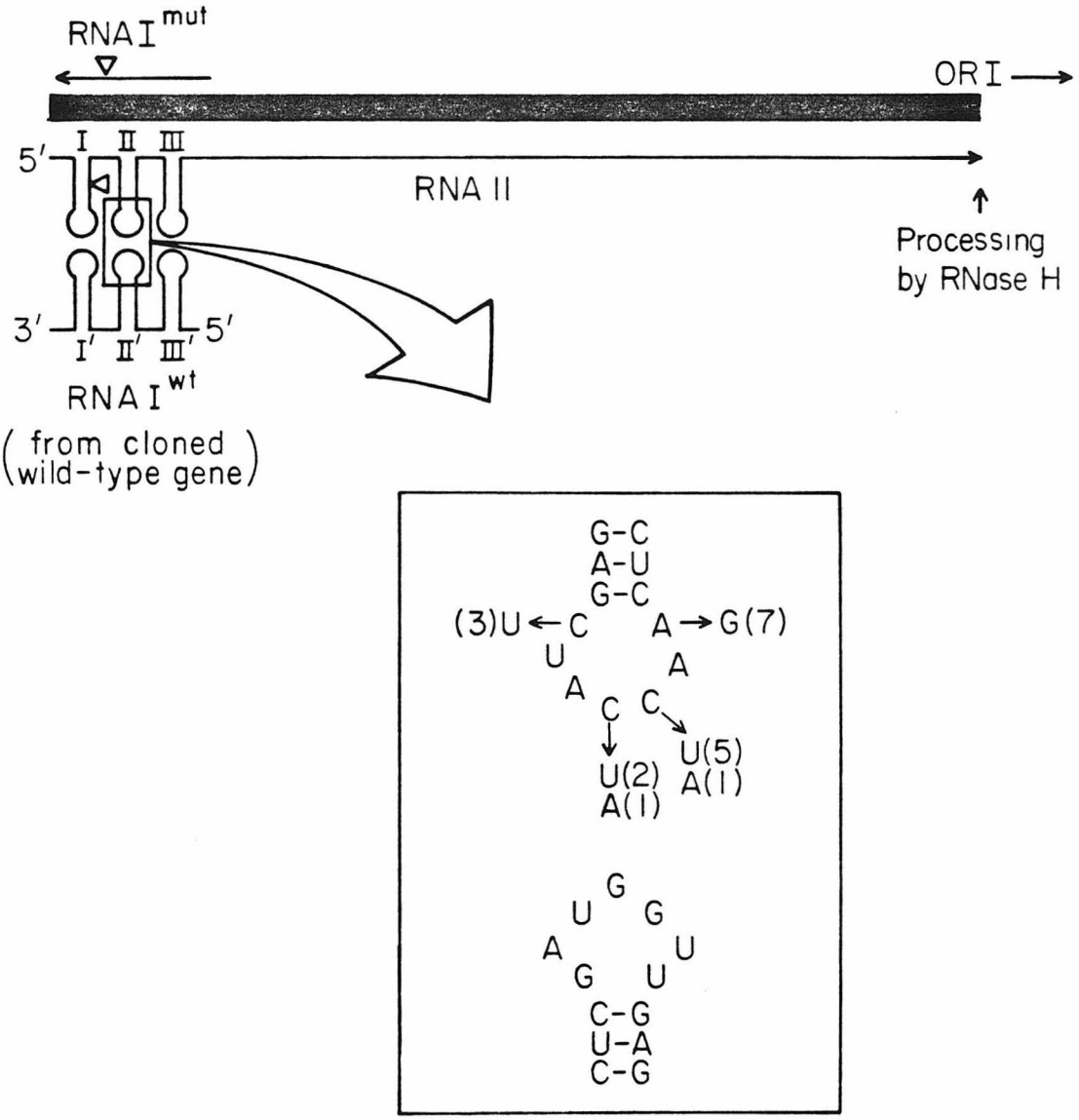
RNA I mut
ORI
5′
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RNA II
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by RNase H
3′
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5′
RNA I wt
(from cloned
wild-type gene)
G-C
A-U
G-C
(3)U ← C
A → G(7)
U
A
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C C
U(5)
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A(1)
G
U G
A U
G U
C-G
U-A
C-G

Figure 1

Figure 1. Nucleotides in RNA II central loop altered by the pDM247 *cop*$^-$ mutations. The number of independent mutants containing a particular mutation is given in parentheses. The locations of the 16 nucleotide insertions in RNA II and the mutant RNA I (RNA I^{mut}) is shown by small triangles.